**UNIVERSIDADE DE LISBOA**

**FACULDADE DE FARMÁCIA**


# NEURO-GLIA-VASCULAR ALTERATIONS BY COMMON NEONATAL CLINICAL CONDITIONS

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# NEURO-GLIA-VASCULAR ALTERATIONS BY COMMON NEONATAL CLINICAL CONDITIONS

# ALTERAÇÕES NA TRÍADE NEURO-GLIA-VASCULAR POR DOENÇAS COMUNS DO PERÍODO NEONATAL

Dissertação apresentada à Faculdade de Farmácia da Universidade de Lisboa para obtenção do grau de Doutor em Farmácia (Biologia Celular e Molecular)

Tese orientada por:
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**Filipa Solange Parreira Lourenço Cardoso**
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# Index

# Figure Index

CHAPTER 4 – TIMING OF NEONATAL SYSTEMIC INFLAMMATION DETERMINES NEURODEVELOPMENTAL OUTCOME AND GLIAL RESPONSE IN YOUNG ADULT MICE



CHAPTER 5 – CONCLUDING REMARKS

# Table Index

# Abbreviations

| | |
|---|---|
| **ABC** | ATP-binding cassette |
| **AJ** | Adherens junctions |
| **Ang** | Angiopoietin |
| **APC** | Antigen presenting cells |
| **ATX** | Autotaxin |
| **BBB** | Blood-brain barrier |
| **BDNF** | Brain-derived neurotrophic factor |
| **Bf** | Free bilirubin |
| **bFGF** | Basic fibroblast growth factor |
| **BMEC** | Brain microvascular endothelial cells |
| **CAM** | Cell adhesion molecules |
| **CCR** | Chemokine receptor |
| **CD** | Cluster of differentiation |
| **CCL** | Chemokine ligand |
| **CNS** | Central nervous system |
| **COX** | Cyclooxygenase |
| **CSF** | Cerebro-spinal fluid |
| **CX3CL1** | Fractalkine |
| **CX3CR1** | Fractalkine receptor |
| **DC** | Dendritic cells |
| **$E_x$** | Embryonic day x |
| **EBA** | Evans' blue bound to albumin |
| **ECM** | Extracellular matrix |
| **ENP** | Early neonatal period |
| **GDNF** | Glial cell line-derived neurotrophic factor |
| **GFAP** | Glial fibrillary acidic protein |
| **GFP** | Green fluorescent protein |
| **GLUT1** | Glucose transporter 1 |
| **GM-CSF** | Granulocyte/macrophage-colony stimulating factor |
| **GW** | Gestational weeks |
| **HMGB1** | High-mobility group box 1 |
| **HSA** | Human serum albumin |
| **Iba1** | Ionized calcium-binding adapter molecule 1 |
| **IFN** | Interferon |

| | |
|---|---|
| **IGF-1** | Insulin-like growth factor 1 |
| **IL** | Interleukin |
| **IRAK** | IL-1 receptor-associated kinase |
| **JAM** | Junctional adhesion molecules |
| **KO** | Knockout |
| **LNP** | Late neonatal period |
| **LPA** | Lysophosphatidic acid |
| **LPS** | Lipopolysaccharide |
| **MBP** | Myelin basic protein |
| **MHC** | Major histocompatibility complex |
| **MMP** | Matrix metalloproteinases |
| **Mrp** | Multidrug resistance proteins |
| **NaF** | Sodium fluorescein |
| **NF-κB** | Nuclear factor-kappaB |
| **NGF** | Neural growth factor |
| **M-CSF** | Macrophage-colony stimulating factor |
| **NMDA** | N-methyl-D-aspartate |
| **NO** | Nitric oxide |
| **nNOS** | Neuronal nitric oxide synthase |
| **NPC** | Neural progenitor cells |
| **NVU** | Neurovascular unit |
| **OPC** | Oligodendrocyte precursor cells |
| **PAMP** | Pathogen-associated molecular patterns |
| **PECAM** | Platelet endothelial cell adhesion molecule |
| **P-gp** | P-glycoprotein |
| **PND** | Postnatal day |
| **PNVP** | Perineural vascular plexus |
| **RAGE** | Receptor for advanced glycation endproducts |
| **ROS** | Reactive oxygen species |
| **SAE** | Sepsis-associated encephalopathy |
| **SMA** | Smooth muscle actin |
| **TEER** | Transendothelial electric resistance |
| **TEM** | Transmission electron microscopy |
| **TGF-β** | Transforming growth factor β |
| **Tie** | Endothelial receptor tyrosine kinase |
| **TJ** | Tight junctions |

| | |
|---|---|
| **TLR** | Toll-like receptor |
| **TNF-α** | Tumour necrosis factor α |
| **TNFR** | Tumour necrosis factor receptor |
| **UCB** | Unconjugated bilirubin |
| **VCAM** | Vascular cell adhesion molecule |
| **VE** | Vascular endothelial |
| **VEGF** | Vascular endothelial growth factor |
| **VEGFR** | Vascular endothelial growth factor receptor |
| ***Wnt*** | *Wingless* |
| **ZO** | *Zonula occludens* |

## Abstract

Neonatal common diseases, such as sepsis and jaundice, may damage the normal development of the central nervous system. Still, the cellular interactions behind the origin of the sequelae remain unknown. Thus, we intended to: (1) investigate the in vitro response of brain microvascular endothelial cells (BMEC), either alone or in the presence of other CNS cells, to stimuli that mimic those diseases– lipopolysaccharide (LPS) for sepsis and unconjugated bilirubin (UCB) for jaundice; (2) to evaluate *in vivo* the neuronal outcome and determine which cellular interactions may be involved, and lastly (3) to dissect if the sequelae depend on the neurodevelopmental stage in which neonatal inflammation occurs.

First, we observed that both LPS and UCB induce damage of mitochondria and of rough endoplasmic reticulum, ultimately leading to cell death in monocultures of rat BMEC. In addition, these molecules cause blood-brain barrier (BBB) breakdown through P-glycoprotein inhibition, activation of matrix metalloproteinases (MMPs), loss of intercellular junctions – transient by LPS and lasting by UCB. Increased permeability and junctional disruption were more severe in co-cultures with astrocytes, particularly in LPS-treated cells. Secondly, newborn mice were treated with LPS during the first week of life (equivalent to human preterm birth) to examine acute-to-delayed effects in the developing neurovascular unit. LPS induced acute loss of body and brain weight, in addition to cerebellar hypoplasia. The latter was linked to neuronal loss/shrinkage, as well as to delayed myelination. Moreover, we observed a late pro-inflammatory response with acute astrogliosis in the parenchyma and microvasculature interface, which was lost at later days after a fast and progressive increase of activated microglia near the vessels. Lastly, we performed further *in vivo* studies to assess the acute effects of LPS-induced neuroinflammation during the second week of life (equivalent to human term birth). Alike in preterm neuroinflammation, we observed neuronal and white matter loss/shrinkage, with increased glial numbers. We further explored the existence of lasting neurodevelopment impairment in young adult age. Interestingly, neuroinflammation during the first week of life led to lasting neuronal loss/shrinkage, whereas neuroinflammation in the second week further induced lasting white matter injury and reduction of astrocytic population. In agreement, when dissecting the effects of priming during the neonatal period, animals with neuroinflammation during the first week had less marked effects compared to an acute young adult response whereas priming during the second week additionally caused neuronal loss and microgliosis.

In conclusion, LPS and UCB act directly on BMEC, impairing BBB integrity which may favor the access of blood-borne molecules into the brain. This increases the risk of altered neurodevelopment, as is the case of neuronal atrophy and delayed myelination observed in our second study. The particular susceptibility of astrocytes to LPS-induced inflammation may not only depend on BMEC response to the stimuli but also on an intricate association with activated microglia. Even though young adults exposed to inflammation in the first week of life recover from the initial loss of astrocytes, they still sustain severe neuronal compromise. On the other hand, young adults subjected to inflammatory stimuli in the second week of life have a more marked demyelination and glial response. These findings contribute to a better understanding of the cellular interactions involved in lasting sequelae observed in sepsis survivors and how they depend on the window of neurodevelopmental stage after birth.

*Keywords*: astrocytes; bilirubin; blood-brain barrier; brain microvascular endothelial cells; lipopolysaccharide; microglia; myelination; neonatal period; neurons; neurovascular unit; systemic inflammation; white matter.

# Resumo

A barreira hemato-encefálica (*blood-brain barrier*, BBB) é uma interface dinâmica entre o sangue e o parênquima encefálico, sendo as células endoteliais da microvasculatura encefálica (*brain microvascular endothelial cells*, BMEC) consideradas a sua base anatómica. As BMEC possuem propriedades únicas que regulam a passagem seletiva de compostos através do endotélio microvascular encefálico, tais como o transportador de efluxo da família ABC glicoproteína-P (*P-glycoprotein*, P-gp) e o transporte transcelular por cavéolas. Além disso, o endotélio é caracterizado por elevada resistência elétrica transendotelial (*transendothelial electrical resistance*, TEER) e baixa permeabilidade paracelular devido aos seus coesos complexos de junções intercelulares, compostos por junções de oclusão (*tight junctions*, TJ) e de aderência (*adherens junctions*, AJ). As BMEC são parte da unidade neurovascular (*neurovascular unit*, NVU) que inclui adicionalmente a membrana basal e outras células do sistema nervoso central (*central nervous system*, CNS), como é o caso das células gliais – essenciais para a indução das propriedades da BBB, função neuronal e resposta imunitária. Assim, condições patológicas que afetem o funcionamento da NVU diminuem a função da BBB, originando ou favorecendo danos.

Entre as condições clínicas neonatais mais comuns encontram-se a icterícia e a sépsis. A icterícia caracteriza-se por uma pigmentação amarela da pele devido à deposição nos tecidos de bilirrubina não conjugada (*unconjugated bilirubin*, UCB) formada pelo catabolismo do grupo hemo, maioritariamente da hemoglobina. A acumulação de UCB no encéfalo representa um risco de neurotoxicidade podendo levar ao aparecimento de encefalopatia bilirrubínica. Entre os diversos fatores de risco da icterícia neonatal encontra-se a sépsis, uma resposta sistémica inflamatória a uma infeção que pode causar disfunção de órgãos e eventualmente levar à morte. De facto, os nossos estudos anteriores mostram que tanto a resposta inflamatória como a morte celular desencadeadas pela UCB nos astrócitos é exacerbada na presença simultânea de lipopolissacárido (*lipopolysaccharide*, LPS). O LPS é um componente da membrana das bactérias Gram-negativas, frequentemente usado em estudos para mimetizar uma infeção e desencadear uma resposta inflamatória. A ativação do seu recetor tipo-Toll 4 (*Toll-like receptor 4*, TLR4) nas BMEC origina a libertação de fatores pró-inflamatórios para o parênquima encefálico culminando em neuroinflamação. Os seus efeitos diretos sobre as BMEC podem ser exacerbados através da resposta inflamatória de outros tipos celulares, desencadeada quer pelo próprio LPS, quer pelas vias pró-inflamatórias ativadas. Este é o caso das proteínas *high-mobility group box 1* (HMGB1) e autotaxina (ATX) que se encontram associadas a microgliose e podem levar à produção de

fatores neurotóxicos, e consequentemente a neurodegeneração. De realçar que as crianças que sobrevivem à sépsis apresentam geralmente perturbações cognitivas e musculares a longo termo, indicando que poderão estar envolvidos danos neuronais.

Esta tese teve como objetivos avaliar: (i) a resposta das BMEC a estímulos que mimetizam a icterícia e a sépsis neonatal *in vitro* (LPS e UCB), em monocamada e em co-cultura com astrócitos (**Capítulo 2**); (ii) as consequências da administração de LPS em murganhos ao nível das interações na NVU neonatal (**Capítulo 3**); e (iii) se as sequelas duradouras dependem da fase de desenvolvimento do período neonatal em que a inflamação sistémica ocorre e, ainda, quais os reflexos de uma sensibilização no período neonatal (*priming*) na resposta resultante de uma nova exposição numa fase mais tardia (**Capítulo 4**).

Iniciámos o nosso estudo *in vitro* determinando os efeitos diretos do LPS e da UCB no endotélio microvascular encefálico de rato. Tanto o LPS como a UCB induziram a morte celular das BMEC, assim como inibição da P-gp e ativação de metaloproteinases (*matrix metalloproteinases*, MMP). Verificou-se ainda a existência de danos ao nível do reticulo endoplasmático e das mitocôndrias por ambos os estímulos. Em células tratadas com LPS foi ainda notório o aumento de invaginações semelhantes a cavéolas e o enfraquecimento das junções intercelulares, ao passo que em células tratadas com UCB a perda das junções intercelulares foi ainda mais marcada. Qualquer uma das moléculas levou ao aumento da permeabilidade endotelial e à diminuição da TEER, tanto em monoculturas como em co-culturas com astrócitos. Observaram-se ainda alterações ao nível da integridade e localização das proteínas de TJ (*zonula occludens*-1 e claudina-5) e AJ (β-catenina). Em geral, os efeitos diretos da UCB sobre as BMEC sofreram agravamento ao longo do tempo, enquanto os danos causados pelo LPS foram reversíveis em monoculturas de BMEC. Curiosamente, em co-cultura com astrócitos os danos induzidos pelo LPS na monocamada continuaram a observar-se a tempos mais longos. Assim, o LPS e a UCB exercem efeitos tóxicos diretos sobre as BMEC, embora com mecanismos de ação e perfis temporais distintos. O enfraquecimento da integridade do endotélio encefálico após exposição a estas moléculas pode aumentar desta forma o risco de lesão, pelo que o tratamento clínico adequado da sépsis e da icterícia no período neonatal são requisitos fundamentais para diminuir ou evitar as sequelas delas decorrentes.

De seguida explorámos mais aprofundadamente os efeitos resultantes de uma inflamação por LPS na primeira semana de vida, em ratinhos CD1 *wild-type* e transgénicos C57BL/6 CX3CR1$^{gfp/+}$. Verificámos haver perda de peso corporal e encefálico, acompanhada de hipoplasia cerebelar. A administração sistémica de LPS

causou a redução da densidade neuronal e sua atrofia, juntamente com um atraso da mielinização. Esta alteração na formação de mielina parece não ser causada por um aumento da atividade fagocítica da microglia, mas sim por inibição ou atraso da diferenciação de células NG2. Verificou-se ainda a existência de resposta pró-inflamatória com aumento da atividade e da expressão de biomarcadores inflamatórios, assim como a presença de astrogliose (células $GFAP^+$) quer no parênquima encefálico quer na interface da microvasculatura. Contudo, os elevados níveis de células $GFAP^+$ diminuem a tempos mais longos, ao mesmo tempo que ocorre um aumento rápido e progressivo de microglia ativada $CX3CR1^{gfp/+}$ junto à microvasculatura. O nosso estudo mostrou assim que, para além do enfraquecimento da BBB, o LPS causa atrofia neuronal e retarda a mielinização, onde a íntima e complexa relação entre astrócitos e microglia se encontra envolvida.

Para melhor avaliar os efeitos duradouros das alterações observadas no período neonatal, e de que modo estes dependem do dano inflamatório ser causado na primeira ou na segunda semana de vida, determinámos as consequências destes danos em animais CD1 no período correspondente a jovens adultos. Primeiramente, avaliámos a resposta do CNS no dia seguinte a um estímulo inflamatório induzido na segunda semana de vida. Verificámos a existência de perda e atrofia neuronais, além de uma acentuada redução de substância branca para a qual poderá contribuir a desmielinização. O aumento da atividade e expressão dos biomarcadores inflamatórios, MM-9 e HMGB1, estão associados a um aumento no número de astrócitos (células $GFAP^+$) e de microglia (células $Iba1^+$), apesar dos níveis de TLR4 se terem mantido inalterados. De seguida avaliámos os danos duradouros em animais jovens adultos após indução de inflamação na primeira ou segunda semana de vida. Os animais com neuroinflamação na primeira semana apresentaram perda e atrofia de neurónios, enquanto os jovens adultos com neuroinflamação durante a segunda semana de vida apresentaram maioritariamente redução da substância branca e perda de astrócitos. Por último avaliámos a resposta a um novo estímulo de LPS em jovens adultos com *priming* durante o período neonatal, relativamente a jovens adultos que apenas receberam a injeção de LPS pela primeira vez enquanto adultos (resposta aguda adulta). Tal como verificado nos efeitos prolongados, os neurónios dos animais com *priming* na primeira semana de vida foram mais vulneráveis do que os animais sujeitos a *priming* durante a segunda semana, levando à atrofia e perda dos mesmos. Para além disso, o *priming* na primeira semana de vida levou a reduções ao nível das camadas neuronais do cerebelo (molecular, de Purkinje e granular), enquanto o *priming* na segunda semana induziu tanto a redução da camada granular como da substância branca. A expressão e atividade dos biomarcadores inflamatórios da

resposta com *priming* na primeira semana de vida mantiveram, na sua maioria, níveis abaixo dos observados na resposta aguda adulta, à exceção da sobreexpressão de ATX. Contrariamente, o *priming* durante a segunda semana de vida manteve níveis semelhantes aos da resposta aguda adulta, e com sobreexpressão tanto de ATX como de HMGB1. Por último avaliámos a proliferação de astrócitos e microglia. O *priming* na primeira semana induziu menor proliferação/migração de microglia relativamente à resposta adulta aguda, e com número de astrócitos semelhante. Curiosamente, o *priming* na segunda semana de vida resultou em números de microglia e astrócitos semelhantes à resposta aguda adulta, excetuando no hipocampo onde se verificou um decréscimo. Assim, este estudo mostra que os danos neuronais são mais marcados em jovens adultos com uma resposta inflamatória na primeira semana de vida (e sem estímulos subsequentes), enquanto as respostas da glia e da substância branca são mais notórias em jovens adultos com *priming* durante a segunda semana de vida. Esperamos que estudos futuros possam elucidar se a modulação dirigida à resposta inflamatória em ratinhos recém-nascidos, feita de modo diferente para a primeira ou segunda semana de vida, é capaz de prevenir as sequelas cerebrais da condição de sépsis neonatal no jovem adulto.

No seu conjunto, estes resultados mostram que a sépsis e a icterícia neonatais comprometem a integridade endotelial levando à quebra da BBB, quer diretamente quer por efeitos indiretos provenientes de outras células do CNS. Nos vários estudos, os astrócitos revelaram ser particularmente sensíveis aos estímulos, e a microglia surgiu como um provável interveniente fundamental na regulação da resposta. Os danos a nível neuronal e da substância branca induzidos pelo LPS no período neonatal mantêm-se pelo menos até à idade de jovem adulto. De realçar que a resposta glial aquando de um segundo estímulo inflamatório na fase de jovem adulto difere consoante o *priming* tenha sido realizado durante a primeira ou a segunda semana de vida. Estes resultados contribuem para a clarificação dos mecanismos determinantes de acrescida vulnerabilidade pelo envelhecimento, dependente da fase de neurodesenvolvimento sujeita a sépsis, favorecendo o desenvolvimento de novas terapêuticas dirigidas aos alvos e determinantes envolvidos em cada uma das duas primeiras semanas de vida, que consigam prevenir ou diminuir as sequelas dela decorrentes.

*Palavras-chave:* astrócitos; barreira hemato-encefálica; bilirrubina; células endoteliais da microvasculatura encefálica; inflamação sistémica neonatal; período neonatal; microglia; neurónios; mielinização; substância branca; unidade neurovascular.

*Chapter 1*

# GENERAL INTRODUCTION

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**Part of this introduction (2.1. Components of the neurovascular unit) was entirely published in the review**

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# 1. Development of the Central Nervous System

In the embryonic development, the central nervous system (CNS) derives originally from the neural plate. This is a thickened region of ectoderm germ cell layer, which later is transformed into the neural tube. In higher vertebrates such as mammalians, the plate is shaped and folded into neural folds that fuse in the midline, thus creating the neural tube. In mice, this process called primary neurulation occurs around embryonic day (E)8 and is completed at E9.5. The spinal neurulation ends the primary neurulation at E10. As for the human embryo, the folding of the neural plate begins 17–18 days after fertilization and is completed at 26–28 days post-fertilization. In the second neurulation, the mesenchymal cells in the tail bud are condensed into an epithelial rod, creating a secondary neural tube continuous to the primary in the caudal sacral and coccygeal regions (Engelhardt and Liebner 2014; Greene and Copp 2009). During this period the primitive CNS is colonized by several progenitor cells that will originate the microvasculature, neurons and glia.

## 1.1. Vessel formation, differentiation and maturation

During vertebrate embryo development, the CNS vessel formation may occur by two different mechanisms: vasculogenesis and angiogenesis (Fig. 1.1). Vasculogenesis is the *de novo* formation of embryonic vascular network from angioblasts or stem cells with mesodermal/endodermal origin. On the other hand, angiogenesis is their subsequent expansion and remodeling *via* a mesodermal/ectodermal origin (Hellström et al. 2001).

The ectoderm germ cell layer has no vascular progenitor cells, so the CNS vasculature begins *via* the process of vasculogenesis, around the time of the primary neurulation. Vascularization occurs between E9 and E10 in mouse (Bauer et al. 1993) and between E11 and 13 in rat (term is 19-22 days) (Stewart and Hayakawa 1994). In humans, this process happens between 10 and 13 gestational weeks (GW) (Virgintino et al. 2004). Mesenchymal cells differentiate into endothelial tubes that form a primitive blood vessel network, the perineural vascular plexus (PNVP), that covers the entire surface of the neural tube and from which new blood vessels develop (Daneman et al. 2010; Engelhardt and Liebner 2014). These early vascular buds initially have large diameter, irregular shape (Bolz et al. 1996) as well as fenestrae (Stewart and Hayakawa 1994), though they later become smaller, thinner, regularly shaped and lose their fenestrae.

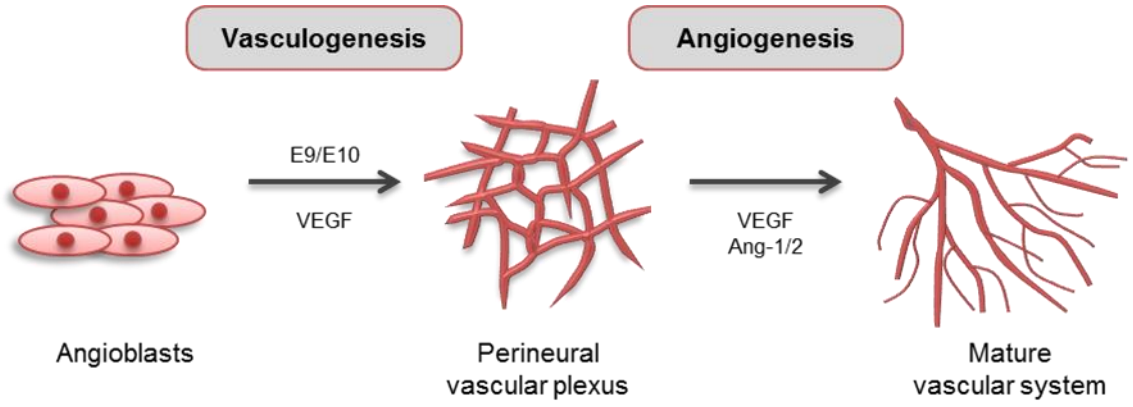


**Figure 1.1 - Development of new vessels in rodent central nervous system.** Around embryonic day (E)9/10 of murine neurodevelopment, the vascular endothelial growth factor (VEGF) induces differentiation and proliferation of endothelial cells from its progenitors (the angioblasts) to form a poorly differentiated perineural vascular plexus (vasculogenesis). Angiopoietin (Ang)-1 and -2 further induce the remodeling of the vascular plexus into a well-structured mature vascular system through endothelial cell sprouting and trimming (angiogenesis), which will later infiltrate the central nervous system.

During angiogenesis, vessel growth might take place through endothelial growth, requiring migration of brain microvascular endothelial cells (BMEC) and tube formation (Ausprunk and Folkman 1977; Risau et al. 1986). Vascular sprouts invade the embryonic neuroectoderm towards a concentration gradient of vascular endothelial growth factor (VEGF)-A, which regulates vessel formation by inducing endothelial proliferation, migration, and tube formation (Greenberg and Jin 2005; Liebner et al. 2011). Its receptors (VEGFR) are also involved in the directing of endothelial sprouting (Darland et al. 2011; Sentilhes et al. 2010). VEGFR-1 is usually associated with primary vascular plexus remodeling and subsequent angiogenesis, whereas VEGFR-2 has been observed in re-inducted angiogenesis, as in pathological conditions, and its inhibition significantly reduces endothelial proliferation (Soares et al. 2013).

Following formation, vessels are stabilized by angiopoietins (Ang) which are specific to the endothelial receptor tyrosine kinase (Tie)-2, and mediate reciprocal interactions between the endothelium and surrounding matrix (Fagiani and Christofori 2013; Liebner et al. 2011). At this stage, Ang-1 and Ang-2 are the two main elements of this family. Ang-1 is mainly produced by mural cells, including pericytes. It mediates vessel maturation by mediating migration, adhesion and survival of endothelial cells (Hellström et al. 2001; Jain 2003; Olsen et al. 2006). It also stabilizes the vessels through the recruitment of mural cells, and makes vessels leak-resistant by VEGF induction (Jain 2003; Thurston et al. 2000). Regarding Ang-2, this molecule is produced by BMEC and it competitively inhibits the binding of Ang-1. Therefore, Ang-2

destabilizes the endothelium and is restricted to areas of vascular remodeling (Jain 2003). Ang-2 has also been shown to antagonize angiogenesis induced by VEGF (Ley et al. 2004).

During angiogenesis BMEC have high paracellular permeability (Liebner et al. 2011), thus these cells acquire several specific properties during the differentiation phase that provides them barrier properties, as early as E12 in mice (Daneman et al. 2010). This is the case of the intercellular junctions' proteins that tightly connect neighboring BMEC, the tight junctions (TJ), which we will further discuss in this introduction. These proteins observed at E12 in mice (Bauer et al. 1993) are observed in humans at 10 GW (Møllgård and Saunders 1986), appearing adult-like by 18 GW (Virgintino et al. 2004). As it will also be discussed later in this introduction, BMEC have specific transporters that endow them the ability to control the passage of molecules to and from the brain. Also at murine E12 it is observed the expression of the glucose transporter (GLUT)1 (Daneman et al. 2010). The efflux ATP-binding cassette (ABC) transporters are expressed at low levels during embryogenesis but increase during human gestational development, matching the adult brain at the time of term birth (Daood et al. 2008).

In the weeks that follow, the vessels' maturation takes place with the recruitment of pericytes and astrocytes for a closer contact (Liebner et al. 2011), in addition to the synthesis of the basement membrane. Studies have shown that pericytes contribute to the formation of TJ by inhibiting the expression of molecules that increase vascular permeability and immune cell infiltration (Daneman et al. 2010). They are recruited to the nascent vessels at very initial stages of development, originating from the local and distal mesenchyme (Sartore et al. 2001), as well as from common BMEC progenitors. The embryonic stem cells will differentiate into BMEC or to vascular smooth muscle cells depending on the stimulation by VEGF or by platelet derived growth factor (PDGF)-β, respectively (Yamashita et al. 2000). Pericytes proliferate and migrate during vascular maturation (Hellström et al. 2001), being associated with endothelial tubes as nascent vessels are generated (Daneman et al. 2010; Tallquist et al. 2003).

Both BMEC and pericytes promote vessel maturation by producing extracellular matrix (ECM) molecules and facilitating the assembly of the matrix (Chambers et al. 2003; Jain 2003). Indeed, as angiogenesis stops, pericytes synthesize components of the ECM, such as proteoglycans, collagen, and elastin. The ECM is initially formed at the parenchymal side of pericytes, though it progressively surrounds the pericyte during the development of the basement membrane (Sá-Pereira et al. 2012). The formation of a basement membrane around capillaries is observed as early as E14 in rat cortex and 8 GW in human foetuses, becoming denser and wider in the postnatal

brain (Roediger et al. 2010). The intact basement membrane stabilizes the newly formed vessels and affects the vascular branching pattern (Jain 2003; Sá-Pereira et al. 2012). It will not be before 2–3 weeks of age (in rodents) that this process is halted and the cortex becomes well vascularized and mature (Saunders et al. 2012).

## 1.2. From precursor cells to neurons and glia

Soon after the beginning of vasculogenesis, the neural precursor cells (NPC) begin to differentiate into neurons and glia (Fig. 1.2). It is their "neuron-to-glia switch" that determines how many neurons or glial cells are formed per brain region (Daneman et al. 2010; Freeman 2010). This process is regulated by transcription factors that determine cell fates and specify subtype identities. Neuronal differentiation of cortical NPC takes place following the activation of the pro-neural genes neurogenins (Yuan and Hassan 2014). Murine neurogenesis is initially promoted, starting at E11, being followed by the proliferation of astrocytes and oligodendrocyte precursor cells (OPC) around E17 (Hirabayashi et al. 2009; Namihira et al. 2009).

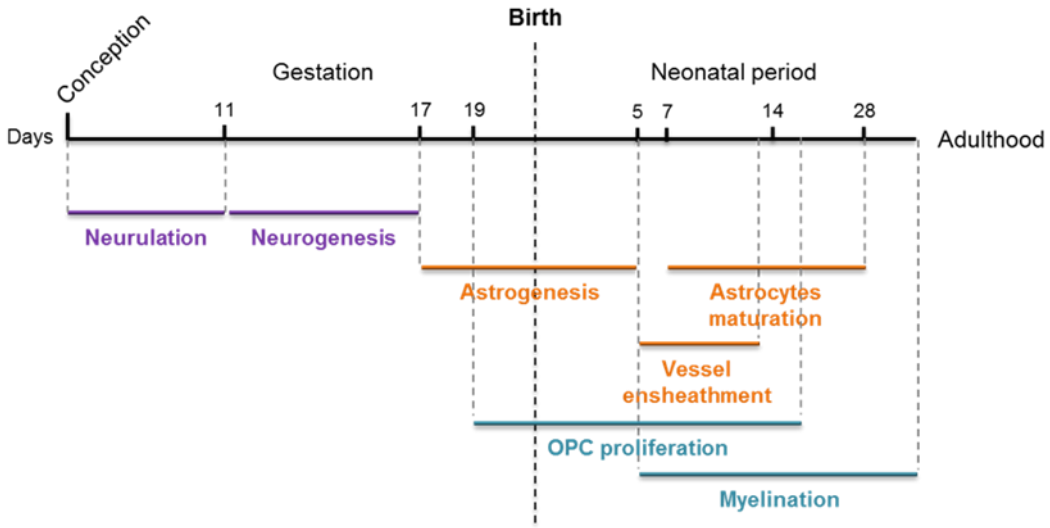


**Figure 1.2 - Timeline of rodent neural precursor cell differentiation.** Following neurulation, neural precursor cells (NPC) go through the process of neurogenesis in order to originate neurons. Later, due to the expression of different factors, NPC undergo astrogenesis forming immature astrocytes that will only fully mature and ensheath the microvessels after birth. Simultaneous to the developing of astrocytes, there is proliferation of oligodendrocyte precursor cells (OPC) that will mature into myelinating oligodendrocytes. Myelination occurs from the postnatal and throughout adulthood.

In the human CNS, there is evidence of contacts between the developing astrocytes and blood vessels as early as 15 GW (Marin-Padilla 1995). However, CNS astrogenesis in rodent occurs directly after birth being induced by cytokines secreted from newborn neurons that promote the expression of glial fibrillary acidic protein (GFAP) in glial precursors (Molofsky et al. 2012). The association of astrocytes with blood vessels is most significant during the first postnatal week, around postnatal day (PND)5 (Daneman et al. 2010; Molofsky et al. 2012; Ullian et al. 2001). Around PND7, their cellular processes are mostly filopodial (i.e., actively growing) and overlap, being trimmed only at the end of the second week of life. By the end of the third week, astrocytic processes ramify while the distal ones become thinner and densely infiltrate the brain tissue, thus turning these cells into a mature appearance (Bushong et al. 2003; Bushong et al. 2004; Freeman 2010).

As for OPC, these are generated during embryogenesis and even in early postnatal life (Miron et al. 2011). They migrate from their sites of origins to colonize the grey matter and the future white matter tracts as early as at murine E19 (Daneman et al. 2010; Miyamoto et al. 2014). OPC that are $NG^{2+}$ cells differentiate into mature myelin-forming oligodendrocytes (Wigley et al. 2007). Initial events in myelination are stimulated by electrical activity in axons, suggesting that electrically active axons will be preferentially myelinated (Wake et al. 2011). Release of glutamate from synaptic vesicles stimulates the development of cholesterol-rich signaling domains between oligodendrocytes and axons, locally increasing the synthesis of myelin basic protein (MBP), the major protein in the myelin sheath. Astoundingly, a single multipolar oligodendrocyte can interact with up to 40 segments on multiple axons (Simons and Trotter 2007; Wake et al. 2011), thus deviations from their normal development may induce lasting neuronal damages.

### 1.3. The “unclosed” origin of microglia

The true origin of microglia has long been discussed, with derivative hypothesis ranging from (1) the outermost connective tissue of the vessels (the vascular adventitia), (2) to the neuroectoderm as oligodendrocytes and astrocytes, (3) to an intrinsic population of hematopoietic stem cells in the CNS, (4) to deriving from circulating monocytes, or even (5) from the peripheral mesodermal/mesenchymal tissues (Chan et al. 2007).

The origin of microglia from monocytes was for years the most accepted hypothesis given that these cells can cross the blood-brain barrier (BBB) into the parenchyma and differentiate into macrophages that are microglia-like. In fact,

macrophages and microglia are quite similar in terms of shared surface markers, receptors, activated phenotypes, turning their molecular distinction into a difficult task (Perego et al. 2013; Vainchtein et al. 2014; Varnum and Ikezu 2012). Nonetheless, microglia exist in normal brain as a stable population which can renew itself (Kettenmann et al. 2011). In addition, a recent study proposes that resident microglia are positive for both the ionized calcium-binding adapter molecule (Iba)1 and the fractalkine (CX3CL1) receptor (CX3CR1), whereas $Iba1^{+}CX3CR1^{-}$ myeloid cells are most likely of peripheral monocyte/macrophage origin (Le Blon et al. 2014). Furthermore, adult microglia express the markers cluster or differentiation (CD)68 and CD204 associated with monocytes and macrophages, but developing microglia do not (Rezaie et al. 2002).

The migration of microglial progenitor cells from the mesodermal/mesenchymal tissues into the CNS is unanimously accepted nowadays (Kettenmann et al. 2011; Nayak et al. 2014) (Fig. 1.3). Most experiments point to the origin of these primitive precursors in the yolk sac, and that their proliferation and differentiation is dependent on several transcription factors and growth factor receptors (Saijo and Glass 2011). In mouse development microglia progenitors migrate from the embryonic yolk sac prior to E8.5, surrounding the neuroepithelium around E9.5. One day later they enter the developing brain *via* the circulatory system (Nayak et al. 2014). In the human brain, microglia colonization coincides not only with vascularization but also with radial glia formation, neuronal migration, and myelination. When colonizing the brain, microglia have an amoeboid morphology rather than the ramified seen in the adult resting cells. In fact, they only become completely ramified in rodents by PND28. In humans, however, ramified microglia are observed in the cortex up to 35 GW (Harry and Kraft 2012; Nayak et al. 2014).

Interestingly, recent studies might resurface the hypothesis of a neuroectoderm origin like oligodendrocytes and astrocytes. It has been shown that after depletion of microglia, the CNS completely repopulates with new microglia through the proliferation of NPC cells that differentiate into microglia (Elmore et al. 2014b). Thus, the true origin of microglia continues to be an open chapter in the development of the CNS.

## 2. Blood-Brain Barrier and the Neurovascular Unit

In 1885, Paul Ehrlich experimentally demonstrated that vital dyes injected into the circulatory system stain all organs of the mammalian body except the brain and spinal cord and attributed this observation to a low affinity of nervous tissue to the dye

(Ehrlich 1885; Ehrlich 1906). About 30 years later, an Ehrlich's student, Edwin Goldmann, noticed the opposite phenomenon by injecting trypan blue directly into the cerebro-spinal fluid (CSF), which stained all CNS and none of the peripheral organs (Goldmann 1913), suggesting the presence of a barrier between the CNS and the circulation. The term *bluthirnschranke* (german for blood–brain barrier), was first used by Lewandowsky (Lewandowsky 1900) while studying the limited permeation of potassium ferrocyanate into the brain. Further studies showed that endothelial tight junctional complexes physically limit solute exchanges between the blood and the brain. This was achieved by injecting horseradish peroxidase intravascularly, showing diffusion between endothelial cells lining skeletal and cardiac vessels, though it did not pass between BMEC in cerebral microvasculature (Reese and Karnovsky 1967).

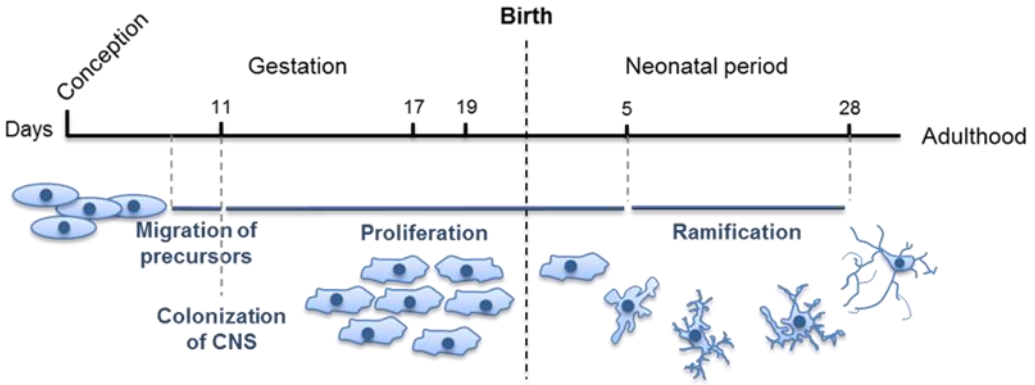


**Figure 1.3 - Timeline of microglia development in rodents.** Microglia precursors migrate from the embryonic yolk sac to colonize the central nervous system (CNS) early in development. These cells will proliferate throughout the CNS during the remaining embryonic development. Their maturation will only occur in the postnatal period, acquiring a fully ramified phenotype by postnatal day 28.

Major BBB functions include: (1) maintenance of CNS homeostasis, (2) protection of brain from extracellular environment, (3) constant supply of nutrients by specific transport systems and (4) direct inflammatory cells to act in response to changes in local environment (Lee et al. 2006; Persidsky et al. 2006; Petty and Lo 2002). Maintenance of homeostasis is achieved through the regulation of ion balance (Hawkins and Davis 2005; Persidsky et al. 2006; Wolburg and Lippoldt 2002) and of compounds influx/efflux (Chaudhuri 2000; Khan 2005). This is essential in protection against harmful substances, variations in blood composition and breakdown of concentration gradients (Kniesel and Wolburg 2000; Petty and Lo 2002; Wolburg and Lippoldt 2002). The BBB is present in all brain regions, except in those regulating

autonomic nervous system and endocrine glands of the body, where blood vessels permit diffusion of molecules across the vessel wall (Ballabh et al. 2004).

## 2.1. Components of the neurovascular unit

In the developing brain, capillaries are differentiated and matured into the BBB (Lee et al. 2006). Only capillary vessels have complete BBB properties, since leakiness increases as the vessel diameter increases (Hawkins and Davis 2005; Marchi et al. 2004). Although BBB permeability itself is controlled by the biochemical properties of BMEC (Pardridge 1999), brain microvascular biology results overall from interactions of these cells with the basement membrane and neighboring glial cells (Kaur and Ling 2008), such as microglia and astrocytes, as well as neurons and perivascular pericytes (Wolburg and Lippoldt 2002; Zlokovic 2008; Zozulya et al. 2008). Altogether these constitute the neurovascular unit (NVU) (Fig. 1.4) (Choi and Kim 2008; Persidsky et al. 2006), essential for both health and function of the CNS (Hawkins and Davis 2005).

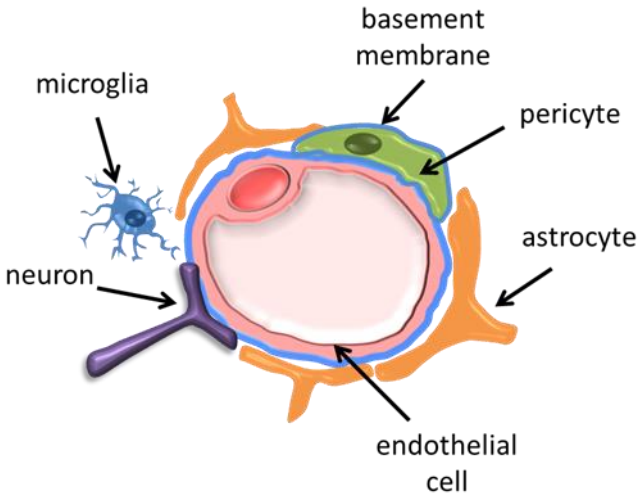


**Figure 1.4 - Schematic overview of the neurovascular unit (NVU).** NVU is composed of endothelial cells, basement membrane, astrocytes and microglia, neurons and pericytes. From (Cardoso et al. 2010).

### *2.1.1. Basement membrane: wrapping the vessels*

The basement membrane is an essential part of the BBB. It surrounds BMEC and engulfs pericytes, anchoring the cells in place and establishing the connection with the surrounding brain resident cells (Carvey et al. 2009). BMEC, pericytes and astrocytes all cooperate to generate and maintain the basement membrane that is constituted by 3 apposed laminas, composed of different ECM classes of molecules (Persidsky et al.

2006; Weiss et al. 2009). These are structural proteins (collagen and elastin), specialized proteins (fibronectin and laminin) and proteoglycans (Adibhatla and Hatcher 2008; Wolburg et al. 2009). The basement membrane also includes matrix adhesion receptors, known as cell adhesion molecules (CAM), as well as signaling proteins, which form an extensive and complex matrix (Carvey et al. 2009). These molecules are expressed in the vascular cells, neurons and supporting glial cells and are essential for maintenance of the BBB. Disruption of the basement membrane can lead to alterations in the cytoskeleton of BMEC, which in turn affects TJ proteins and barrier integrity. Matrix metalloproteinases (MMP) are known to digest the basement membrane, which leads to reduced anchoring of BMEC and matrix–BMEC signaling (Carvey et al. 2009). Accordingly, disruption of ECM has been strongly associated with increased BBB permeability in several pathological conditions (Carvey et al. 2009; Hawkins and Davis 2005; Zlokovic 2008).

### *2.1.2. Neurons in the neurovascular unit*

Little is known about the developmental role that neurons have on the BBB phenotype. However, there is some evidence that neurons can regulate the function of blood vessels in response to metabolic requirements by inducing expression of enzymes unique for BMEC (Persidsky et al. 2006). Also, BMEC and astrocytic processes are directly innervated by noradrenergic, serotonergic, cholinergic, and GABA-ergic neurons, among others (Hawkins and Davis 2005). Moreover, mature endothelium has a reciprocal function in inducing a stable brain microenvironment that enables proper neuronal activity (Choi and Kim 2008).

### *2.1.3. Microglia, patrollers of the central nervous system*

Microglia play a very important role in immune responses of the CNS, surveying local microenvironment and changing the phenotype in response to homeostatic disturbance of the CNS. In fact, these cells present themselves in two forms: resting and activated microglia. When resting, cells have small bodies and long, thin processes; in contrast, activated microglia assume a phagocytic morphology by shifting from long to short processes (Zlokovic 2008). In accordance with the macrophage population in other organs, these cells have diverse effector functions (Ransohoff and Perry 2009). As microglia are found in perivascular space, it is thought that their interactions with BMEC may contribute to the BBB properties (Choi and Kim 2008). Still, the exact mechanisms on how this occurs remain unknown.

### *2.1.4. Pericytes in barrier integrity*

Pericytes (also known as vascular smooth muscle cells, mural cells, or myofibroblasts) are important cellular constituents of the capillaries and post-capillary venules (Dore-Duffy 2008), having a close physical association with the endothelium. They share the same basement membrane with BMEC (Bagley et al. 2005) and cover 22 to 32% of the capillaries (Kim et al. 2006). The location of pericytes on the microvessel and the degree of coverage varies considerably between different microvessel types (Allt and Lawrenson 2001), seeming to correlate with the degree of tightness of the interendothelial junctions (Lai and Kuo 2005). The vascular pericyte synthesizes most elements of the basement membrane including a number of proteoglycans. In addition, pericyte synthesis and release of laminal proteins is thought to be a critical step in the differentiation of the BBB (Dore-Duffy et al. 2006).

Proper association of pericytes with microvascular endothelia is essential to maintain structural support and junctional integrity (Braun et al. 2007; Lai and Kuo 2005). Pericytes and BMEC communicate with each other through several apparatuses such as gap junctions, TJ, adhesion plaques (Allt and Lawrenson 2001) and soluble factors (Bagley et al. 2005).

The association of pericytes to blood vessels has been suggested to regulate BMEC proliferation, migration and differentiation (Persidsky et al. 2006). There is also evidence that pericytes can induce and up-regulate P-glycoprotein (P-gp) functional activity in these cells (Dohgu et al. 2005). Furthermore, interactions between pericytes and BMEC are important for the remodeling and maintenance of the vascular system *via* the secretion of growth factors or modulation of the extracellular matrix. There is evidence that pericytes are involved in the transport across the BBB as well (Allt and Lawrenson 2001). Pericytes make regulatory adjustments in response to stress stimuli, as during severe and prolonged $O_2$ deprivation (Al Ahmad et al. 2009).

Pericytes show rich contents of α-smooth muscle actin, which is a characteristic of the vascular smooth muscle cell, suggesting a contractile ability of pericytes both *in vitro* (Kelley et al. 1987; Shepro and Morel 1993) and *in vivo* (Peppiatt et al. 2006). The physical contacts of pericytes' cellular processes over interendothelial junctions, coupled with the α-smooth muscle actin contents, point to a functional role of pericytes in controlling blood flow (Peppiatt et al. 2006), as well as in regulating junctional permeability (Edelman et al. 2006). Pericytes have been shown to migrate away from brain microvessels in rapid response to hypoxia and traumatic brain injury. Therefore, both of these conditions are associated with increased BBB permeability (Hawkins and

Davis 2005). The disruption of BBB caused by the detachment of pericytes reinforces the idea of pericytes' role in junctional permeability (Nishioku et al. 2009).

### *2.1.5. Astrocytes as inductors of barrier properties*

Astrocytes are glial cells whose endfeet form a lacework of fine lamellae closely apposed to the outer surface of the BBB endothelium and respective basement membrane (Abbott 2002). They play a major role in promoting proteoglycan synthesis with a resultant increase in BMEC charge selectivity and playing an important role in the induction of BBB functions (Bernoud et al. 1998; Yamagata et al. 1997). Astrocytes are also important for proper neuronal function, and the close proximity of neuronal cell bodies to brain capillaries suggests that interactions between all these elements are essential for a functional NVU (Persidsky et al. 2006).

In some areas of the CNS the microvessels lack astrocytic ensheathment but still exhibit some BBB features, which are likely due to soluble factors acting from the glia limitans or the subarachnoid CSF (Abbott 2002). Subsequent studies also showed loss and restoration of barrier integrity *in vivo* following a temporary focal loss of astrocytes (Persidsky et al. 2006; Willis et al. 2004). Attempts to recover BBB properties in BMEC cultures have included co-culturing BMEC with astrocytes and/or astrocyte-conditioned medium (Colgan et al. 2008). Astrocytes may therefore modulate the BBB phenotype without being directly involved in the physical BBB properties. Studies using non-contact co-cultivation with astrocytes revealed that the secretion of factors into the medium contribute to the barrier properties of human BMEC (HBMEC) by up-regulating the tight junctional proteins *zonula occludens* (ZO)-1 and occludin, and reducing the transendothelial permeability across the HBMEC (Colgan et al. 2008; Siddharthan et al. 2007). Interestingly, astrocytes are also able to endow non-neural endothelial cells with BBB properties (Hayashi et al. 1997), whereas enteric glia, which share morphological, biochemical, and functional properties with astrocytes, accelerates the formation of the characteristics of the BBB in spinal cord capillaries (Jiang et al. 2005).

### *2.1.6. Endothelial cells*

BMEC were demonstrated to play a key role in BBB properties as it was observed that horseradish peroxidase could not pass the endothelial layer from either direction, and that brain capillaries from amphibians have high electric impedance, indicative of restriction to the movement of ions, despite the absence of surrounding astrocytes (Hawkins et al. 2006). It is now accepted that the cerebral endothelium forms the

anatomic basis of the BBB in higher animals (Hawkins et al. 2006) and that the capillaries make up the primary part of the BBB (Khan 2005).

BMEC interact intimately with other brain cells of the NVU, and hence can act as mediators between blood and brain (Calabria and Shusta 2008). They regulate the selective transport and metabolism of substances from blood to brain as well as in the opposite direction from the parenchyma back to the systemic circulation (Zheng et al. 2003). The BMEC barrier line is the most critical for preventing toxic substances from entering the brain (Ueno 2007). Communication between BMEC and other surrounding cells enhances the barrier functions consequently resulting in maintenance and elaboration of proper brain homeostasis (Choi and Kim 2008).

The BMEC lining the cerebral capillaries differs fundamentally from other vascular endothelia in their capacity to regulate the passage of molecules and cells to and from the neural parenchyma (Ge et al. 2005; Weksler et al. 2005). The capillary endothelium in the brain is 50-100 times tighter than peripheral microvessels as a result of special properties that cause severe restriction of the paracellular pathway for diffusion of hydrophilic solutes (Abbott 2002). BMEC cytoplasm has uniform thickness with no fenestrae, low pinocytotic activity and a continuous basement membrane (Chaudhuri 2000; de Boer and Gaillard 2006). In addition, BMEC have a negative surface charge that repulses negatively charged compounds (de Boer and Gaillard 2006). They have a greater number and volume of mitochondria compared with endothelium in other organs. These characteristics enhance the energy potential (Persidsky et al. 2006), providing energy for enzymes to break down compounds and allowing various selective transport systems to actively transport nutrients and other compounds into and out of the brain (de Boer and Gaillard 2006).

BMEC are tethered to the basement membrane through focal adhesions, which consist mainly of transmembrane proteins (Kumar et al. 2009) that also participate in intercellular adhesion (Wolburg et al. 2009). The transmembrane proteins have been classified into three families of CAM according to their structure: selectins, immunoglobulin superfamily, and integrins (Lee and Benveniste 1999). In BMEC, integrins play an important role during angiogenesis and in the maintenance of vascular integrity (Wolburg et al. 2009). They function as adhesion receptors, in addition to transmitting chemical signals and mechanical forces between the ECM and the cytoskeleton (Yuan 2002). As in other cell types, the three primary elements of the cytoskeleton are actin filaments, intermediate filaments and microtubules. Actin filaments are composed of globular monomers of G-actin, which polymerize to form helical and asymmetrical filaments of F-actin that form contractile bundles and filamentous networks, essential for the maintenance of cell shape and integrity

(Kierszenbaum 2007). At the BMEC, bands of F-actin are anchored to proteins involved in the adhesion to ECM (Kierszenbaum 2007) and linked to membrane and cytosolic proteins involved in intercellular junctions to form a structure denoted as the actin-rich adhesion belt (Stamatovic et al. 2008), sometimes also referred to as perijunctional actin (Terry et al. 2010).

***Intercellular junctions***

BMEC lining the vascular wall have narrow junctional complexes that eliminate gaps or spaces between cells and prevent any free diffusion of blood-borne substances into the brain parenchymal space (Weiss et al. 2009; Zlokovic 2008). In fact, the cerebral microvasculature lining is characterized by the presence of an elaborated junctional complex that includes mainly TJ and adherens junction (AJ) proteins (Hawkins and Davis 2005) (Fig. 1.5).

*Tight Junctions*

TJ are the main structures responsible for the barrier properties. These are elaborate structures located on the apical region of BMEC. They function both as a seal that regulates lateral diffusion between the apical and basolateral plasma membrane domains, which enables asymmetric distribution of membrane constituents, and as a limit to paracellular permeability (Ge et al. 2005; Hawkins et al. 2005; Hawkins et al. 2006; Persidsky et al. 2006). Studies using TJ from different tissues with varying transendothelial and transepithelial electrical resistances (TEER) figured out a correlation between increased organization of cytoplasmic fibrils and decreased membrane permeability (Huber et al. 2001). The BBB TJ should not be regarded as isolated barrier molecules, but as highly dynamic structures that are under the close regulation of the brain microenvironment (Wolburg et al. 2009).

Another role for TJ as dynamic heteromeric signaling complexes has been unraveled, involving the control of gene expression, cell proliferation and differentiation (Gonzalez-Mariscal et al. 2009). The signaling at TJ is bi-directional, so that signals are transmitted from the cell interior to forming or existing TJ to regulate its assembly and function, whereas TJ coordinately receive and transmit information back to the cell interior to regulate gene expression and subsequent cellular responses (Terry et al. 2010).

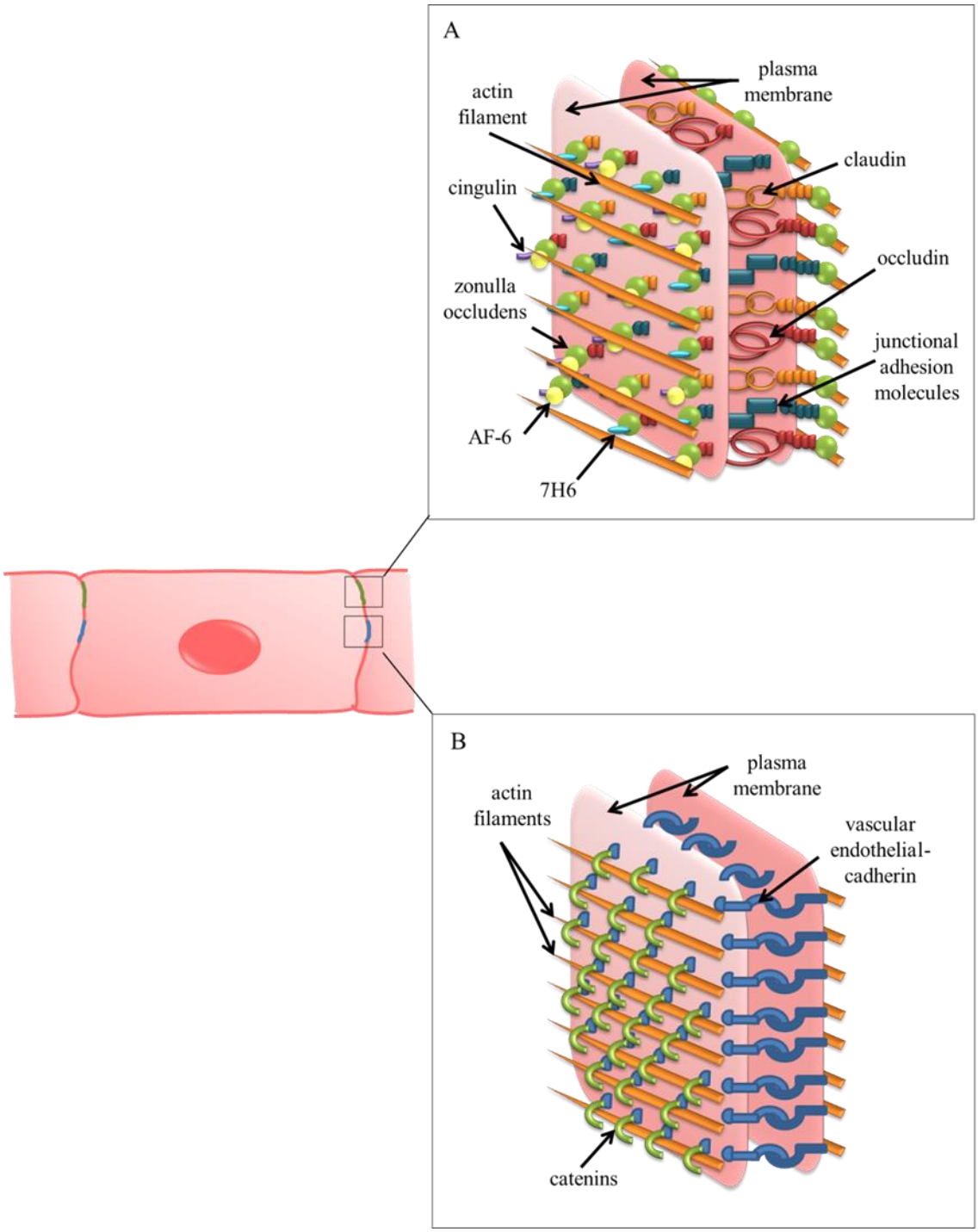


**Figure 1.5 - Endothelial cells at the blood–brain barrier present an elaborated junctional complex formed by tight junctions and adherens junctions.** A, Tight junctions are located on the apical region of endothelial cells and form an intricate complex of parallel, interconnected, transmembrane and cytoplasmatic strands of proteins arranged as a series of multiple barriers. B, Adherens junctions are located below the tight junctions and are composed of transmembrane glycoproteins linked to the cytoskeleton by cytoplasmatic proteins, giving place to the adhesion belt. From (Cardoso et al. 2010).

The BBB TJ of mammalian species is characterized first of all by the highest complexity in the vasculature of the body (Kniesel and Wolburg 2000). Structurally, TJ form an intricate complex of parallel, interconnected, transmembrane and cytoplasmatic strands of proteins arranged as a series of multiple barriers (Wolburg and Lippoldt 2002). The transmembrane proteins are integral membrane proteins that interact with those of neighboring plasma membrane and form the TJ barrier (Petty and Lo 2002). The cytoplasmic TJ accessory proteins are involved in the connection of integral TJ to the actin cytoskeleton (Lee et al. 2004; Persidsky et al. 2006; Petty and Lo 2002; Weiss et al. 2009).

*Claudins*

The claudins are a family of transmembrane TJ proteins that seem to fulfill the task of establishing barrier properties (Ueno 2007; Zlokovic 2008). These proteins are integral membrane proteins that form dimers and bind homotypically to other claudin molecules in adjacent BMEC (Huber et al. 2001; Petty and Lo 2002) and are believed to be responsible for permeability restriction (Tsukita et al. 2001). Although overexpression of claudins can induce cell aggregation and formation of TJ-like structures (Yamamoto et al. 2008), occludin expression does not result in the TJ formation. Thus, it appears that claudins form the primary seal of the TJ, and occludin acts as an additional support structure (Persidsky et al. 2006).

The claudins are not randomly distributed throughout the organs, appearing to be expressed in a tissue-specific manner. Claudin-1 and claudin-5 are associated with maintenance of normal BBB function (Vorbrodt and Dobrogowska 2003) and are important in angiogenesis and in disease processes with increased vessel permeability (Wolburg and Lippoldt 2002). In line with the role of claudin-5 in permeability, claudin-5 knockout mice are characterized by a size-selective BBB defect (Nitta et al. 2003), whereas down-regulation of claudin-5 expression correlates with breakdown of the BBB (Argaw et al. 2009). Lately, new functions of claudin family proteins in response to cellular stress (Romanitan et al. 2010), as well as in the regulation of embryonic morphogenesis (Gupta and Ryan 2010) were suggested.

*Occludin*

The transmembrane occludin is highly expressed and consistently stains in a continuous pattern along the cell margins in the cerebral endothelium, whereas it is much more sparsely distributed in non-neural endothelium (Hawkins and Davis 2005). It has been shown that high levels of occludin ensure decreased paracellular permeability (Huber et al. 2001) and high TEER of the BMEC monolayers (Persidsky et

al. 2006), having therefore an active role in BBB function (Yamamoto et al. 2008). However, it must be seen more in a regulatory context than as a major structural protein in the establishment of the barrier properties (Wolburg and Lippoldt 2002). Results of several knockout and knockdown experiments indicate that occludin is not essential for the formation of TJ (Hawkins and Davis 2005) and that normal expression and localization of other junctional proteins compensate for occludin loss (Zlokovic 2008). The cytoplasmic C-terminal domain provides the connection of occludin with the cytoskeleton via accessory proteins (Shimizu et al., 2008).

*Junctional adhesion molecules*

The JAM family consists of JAM-1, JAM-2, and JAM-3. These transmembrane proteins mediates homophilic and probably also heterophilic interactions in the TJ (Stamatovic et al. 2008; Vorbrodt and Dobrogowska 2003; Wolburg and Lippoldt 2002). A tissue-specific distribution of JAM was observed, reflecting the involvement of these proteins in different interendothelial junctions. In fact, JAM-1 is predominantly expressed in brain (Aurrand-Lions et al. 2001; Bernacki et al. 2008), accounting for the network of TJ strands unique to the brain vascular system, whereas JAM-2 is highly expressed in lymphatic endothelial cells, and JAM-3 is found in most endothelial contacts ranging from brain vasculature to high endothelium venules (Aurrand-Lions et al. 2001). Interestingly, JAM are also expressed in circulating monocytes, neutrophils, lymphocyte subsets, and platelets, as well as in dendritic cells (Ogasawara et al. 2009; Ueno 2007). JAM-1 is involved in cell-to-cell adhesion, organizing the tight junctional structure, and taking part in the formation of TJ together with occludin and claudins (Vorbrodt and Dobrogowska 2003). JAM also play a role in developmental processes and regulate the transendothelial migration of leukocytes (Persidsky et al. 2006; Stamatovic et al. 2008). Although the functions of JAM are still largely unknown in mature BBB, it has been suggested that altered expression of JAM-1, in addition to affecting the junctional tightness, may also disturb leukocyte trafficking, with implications for immune status within the diseased CNS (Zlokovic 2008).

*Cytoplasmatic proteins*

Submembrane TJ-associated proteins such as ZO proteins (ZO-1, ZO-2 and ZO-3) serve as recognition proteins for TJ placement and as a support structure for signal transduction proteins (Huber et al. 2001). ZO-1 is mostly expressed in endothelial and epithelial cells that normally form the TJ assembly (Kniesel and Wolburg 2000; Persidsky et al. 2006). Though it is also expressed in other cell types that do not form TJ, there is no TJ without ZO-1. ZO-1 molecules are located in the cytoplasmic side of

the BMEC plasma membranes delimiting the interendothelial cleft (Vorbrodt and Dobrogowska 2003) and connecting transmembranous TJ proteins with the actin cytoskeleton. Loss or dissociation of ZO-1 from the junctional complexes is associated with increased barrier permeability (Choi and Kim 2008). ZO-2 has significant homology to ZO-1 and it was thought to be restricted exclusively to the TJ region (Kniesel and Wolburg 2000), however, it has also been found in non-TJ containing tissues. ZO-2 is not only an extremely important structural protein, but also a nuclear factor influencing gene expression (Wolburg et al. 2009) and blocking cell cycle progression (Gonzalez-Mariscal et al. 2009). ZO-3 binds occludin and ZO-1 directly, but not ZO-2 (Kniesel and Wolburg 2000). The complex formed by ZO proteins is considered to be involved in cadherin-based cell adhesion through their binding to α-catenin and to actin filaments. In adverse conditions, as chemical stress or mechanical injury, ZO-1 and ZO-2 concentrate at the nucleus and associate with proteins involved in the regulation of gene transcription and cell proliferation (Gonzalez-Mariscal et al. 2009).

Other cytoplasmic proteins are cingulin, AF-6 and 7H6. Phosphoprotein cingulin is located at the cytoplasmatic side of TJ as well. It connects to ZO and JAM proteins, AF-6 and myosin, implying a role as a scaffold between transmembrane proteins and the cytoskeleton (Cordenonsi et al. 1999; Sandoval and Witt 2008). There are studies that also imply a role of cingulin in regulation of TJ permeability (Cordenonsi et al. 1999). 7H6 phosphoprotein seems to be correlated with TJ impermeability to ions and large molecules. There is evidence that this protein may detach from TJ when ATP levels decrease, resulting in increased paracellular permeability (Bernacki et al. 2008; Mitic and Anderson 1998).

*Adherens Junctions*

Below the TJ, in the basal region of lateral plasma membrane there are AJ that give place to a continuous belt – the adhesion belt (Petty and Lo 2002). AJ mediate the adhesion of BMEC to each other, the contact inhibition during vascular growth and remodeling, the initiation of cell polarity, and the regulation of paracellular permeability (Hawkins and Davis 2005), in addition to contributing to the barrier function (Carvey et al. 2009). Cell–cell adhesion through actin filaments linking (Cook et al. 2008) involves VE-cadherin and catenins, constituents of AJ (Perrière et al. 2007). The cadherin family and their intracellularly associated catenin proteins form complexes of central importance to the sorting and morphogenic processes of developing animal tissues and in maintaining the integrity and identity of adult tissues (Tao et al. 1996).

*Cadherins*

$Ca^{2+}$-dependent cell–cell adhesion is dependent on a family of transmembrane glycoproteins named cadherins (Navarro et al. 1998), which present a certain degree of cell type specificity. VE-cadherin, also known as cadherin-5, is an integral membrane glycoprotein expressed exclusively in cells of vascular epithelial origin, whereas neural (N)-cadherin is expressed in cells of the nervous tissue, vascular smooth muscle cells, and myocytes (Navarro et al. 1998). VE-cadherin is an important determinant of microvascular integrity both *in vitro* and *in vivo* (Vorbrodt and Dobrogowska 2003). It clusters at cell junctions and mediates cell adhesion, inhibits cell proliferation, and decreases cell permeability and migration when overexpressed in various cell types (Cook et al. 2008). All cadherins contain a plasma membrane-spanning domain and a cytoplasmic domain associated with catenins, other molecular components of the junctional complex (Vorbrodt and Dobrogowska 2003). VE-cadherin's expression at cell junctions, however, is independent of β-catenin binding, which appears to be required only for junction stabilization (Cook et al. 2008).

*Catenins*

Catenins were first characterized as linking the cytoplasmic domains of cadherin cell–cell adhesion molecules to the cortical actin cytoskeleton. Catenins' major role is to anchor the cadherin complex to the actin cytoskeleton but they also participate in cell and developmental signaling pathways (Stamatovic et al. 2008). There are four types of catenin proteins: α-, β-, δ- and γ-catenin. α- and β-catenin are located in interendothelial junctions of BBB-type brain capillaries and their expression is required for cadherins to work as adhesion molecules (Vorbrodt et al. 2008). β-catenin is essential in BMEC for normal vascular patterning. It is a structural protein that participates in cell–cell adhesion and is also involved in the *Wingless* signaling pathway and in gene expression (Cook et al. 2008; Vorbrodt et al. 2008), serving as a mediator in regulation of P-gp and other multidrug efflux transporters in brain vasculature (Lim et al. 2008). In addition, β-catenin is linked to the cell membrane in a complex with VE-cadherin and platelet–endothelial cell adhesion molecule (PECAM-1 or CD31). This adhesion molecule mediates homophilic adhesion (Wolburg et al. 2009). Upon stimulation with growth factors this membrane associated complex is dissociated, releasing another source of β-catenin for movement to the nucleus and transcription (Petty and Lo 2002). It is possible that the up-regulation of β-catenin accounts for the maintenance of TJ protein assembly and barrier function (Vorbrodt et al. 2008). δ-catenin has been implicated as a regulator of the NF-κB transcription factor (Perez-

Moreno et al. 2006), while γ-catenin is closely related to β-catenin and can substitute it in the cadherin–catenin complex (Vorbrodt and Dobrogowska 2003).

***Main pathways across the Blood-Brain Barrier***

The BBB is more than an impermeable wall. BMEC have a unique pattern of receptors and specific transport systems that facilitate the uptake of important nutrients and hormones, in addition to active pumps that help to regulate the concentrations of ions, metabolites and xenobiotics in the brain (Weksler et al. 2005; Zheng et al. 2003; Zlokovic 2008). The BMEC are able to efficiently supply the brain with the metabolites required while contributing to the maintenance of the brain's ionic homeostasis and protecting the CNS from a large variety of potentially harmful hydrophobic compounds (Betz 1992; Choi and Kim 2008). Passage of molecules across BMEC can occur between adjacent cells (the paracellular pathway) or through the cells (the transcellular pathway) (Pardridge 1999). In the endothelium, the relationship of paracellular and transcellular permeability is of crucial importance for the regulation of overall transendothelial permeability (Wolburg et al. 2009). When it comes to the paracellular passage, ions and solutes diffuse between adjacent cells according to their concentration gradient (Petty and Lo 2002). As for the transcellular pathway, it involves different mechanisms including passive diffusion of lipophilic compounds, receptor-mediated shuttling and transcytosis. Small lipophilic molecules, such as oxygen, $CO_2$ and ethanol (Abbott 2002), can pass the BBB freely by diffusion (Zheng et al. 2003), whereas hydrophilic molecules, such as peptides and proteins, may enter the brain through specific transport mechanisms (Norsted et al. 2008). For that, an elaborate system of transport proteins such as GLUT1 and ABC transporters, among others, is expressed on the luminal and abluminal BMEC membranes (Hawkins et al. 2002). Polar and lipid-insoluble molecules do not cross the BBB (Scherrmann 2002).

*Caveolae*

Caveolae are sites of endothelial transcytosis, endocytosis, signal transduction, and act as docking sites (Simionescu et al. 2002; Wolburg et al. 2009). Caveolae are dynamic pieces of membrane that are either opened for receiving and releasing material or closed for processing, storage, and delivery to the cell (Anderson 1993). These invaginations of the plasma membrane are rich in the structural and functional coat protein caveolin (Fig. 1.6). Caveolin-1 is the major structural protein of caveolae and is involved in various aspects of vesicular trafficking and signal transduction pathways (Huber et al. 2001). In the BBB, the presence of both caveolin-1 and -2 in microvessels was demonstrated for BMEC of rats, rhesus monkeys, porcine and of

normal human brain samples (Virgintino et al. 2002). Caveolin-1 appears to act not as a determinant of caveolae invagination and internalization but rather as a regulator that stabilizes caveolae at the plasma membrane and reduces the endocytic potential of caveolae domains (Nabi and Le 2003). Caveolin-1 also appears to constitute an early and critical modulator that controls signaling pathways leading to the disruption of TJ proteins (Zhong et al. 2008) as indicated by the localization of caveolae close to junctional cell contacts and the fact that both occludin and ZO-1 are organized within TJ by association with caveolin-1 (Smith and Gumbleton 2006).

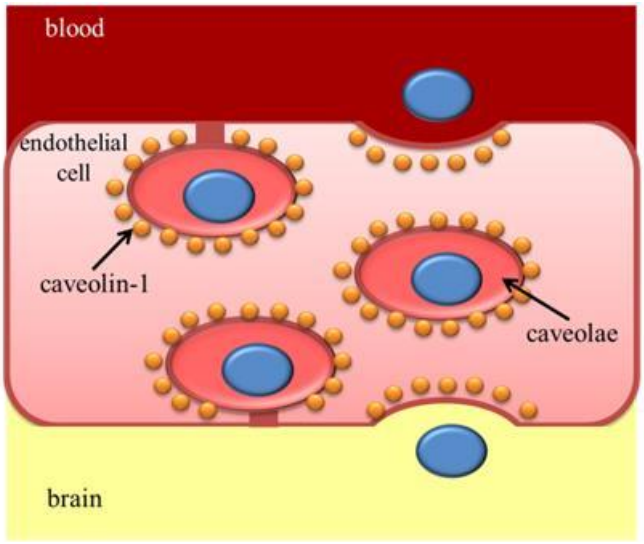


**Figure 1.6 - Caveolin-1 is the major structural protein of caveolae.** Caveolae are involved in various aspects of vesicular trafficking and signal transduction pathways. From (Cardoso et al. 2010).

*Glucose transporter-1*

GLUT1 ensures nutrient delivery, supplying glucose for the brain (Fig. 1.7) (Persidsky et al. 2006), which is its main energy source. GLUT1 is highly restricted to BMEC (Wolburg et al. 2009) with higher density at the abluminal membrane than at the luminal. The asymmetrical distribution provides homeostatic control for glucose influx into the brain by preventing accumulation at levels higher than in the blood (Zlokovic 2008). Interestingly, GLUT1 may also be present at peripheral nerve pericytes transporting D-glucose from the circulating blood into the brain and peripheral nervous parenchyma in cooperation with that on the BMEC (Shimizu et al. 2008).

*ATP-binding cassette transporters*

ABC transporters, as P-gp and the multidrug resistance associated proteins, have the potential to reduce the penetration of many drugs into the brain and increase their efflux from the brain (Fig. 1.7). P-gp is a transporter for the acquisition of the multidrug resistance phenotype, as it prevents the entry of blood-borne substances into the brain and facilitates their transport out of brain parenchyma (Choi and Kim 2008). The expression of this efflux transporter among blood–brain interface is still under investigation. In fact, although P-gp in the brain has been thought to be primarily located in the apical or luminal membranes of BMEC (Gazzin et al. 2008; Virgintino et al. 2002), it was also reported that such protein localizes to both the luminal and the abluminal membranes of the BMEC (Bendayan et al. 2006). These apparently contradictory findings can be reconciled by the recent studies of Tai et al. (2009), which showed that the transporter is present in both membranes but P-gp molecules associated with the apical membrane are nearly 3 times greater than that on the basolateral membrane. The localization of the transporter in both poles would permit that the efflux of molecules from the brain endothelium can be initiated at the luminal membrane, whereas the elimination of drugs from the brain occurs due to the abluminal membrane P-gp that acts in concert with the luminal transporter. It appears to exist an association between P-gp and caveolin-1, as the down-regulation of caveolin-1 enhances the transport activity of P-gp (Barakat et al. 2007; Demeule et al. 2000). P-gp is also expressed at the endothelial subcellular level along the nuclear envelope and in caveolae, cytoplasmic vesicles, Golgi complex, and rough endoplasmic reticulum (Bendayan et al. 2006; Tai et al. 2009). In addition to EC, P-gp is also expressed at the plasma membrane of both pericytes and astrocytes, suggesting that this glycoprotein may regulate drug transport processes in the entire BBB (Bendayan et al. 2006).

## 2.2. Crucial interactions within the developing neurovascular unit

During embryonic and fetal development there are fundamental crosstalks and interdependence between the vascular and the neuronal systems. Integrated responses in neurons, glia, brain blood vessels, and circulating peripheral cells all contribute to the pathogenesis of injury and mechanisms of repair after stroke and trauma (Hayakawa et al. 2014).

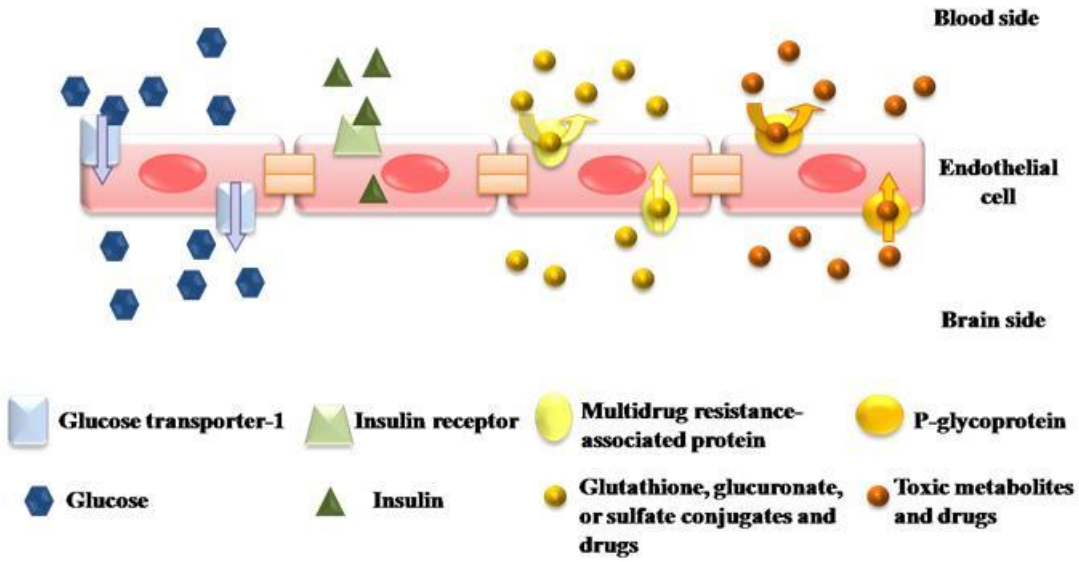


**Figure 1.7 - Endothelial cells comprise a large variety of specific transporters and receptors, which regulate brain concentrations of nutrients, hormones, metabolites, and xenobiotics.** For simplicity, only the glucose transporter-1, the insulin receptor and the ABC transporters, as the multidrug resistance-associated protein and the P-glycoprotein, and respective substrates, are represented. From (Cardoso et al. 2010).

### *2.2.1. The neurovascular coupling*

The neurovascular coupling describes increased regional cerebral blood flow in response to physiological neuronal activation and decreased regional cerebral blood flow in response to neuronal deactivation (Fig. 1.8) (Yao and Tsirka 2014). This is due to the fact that neuronal activation causes glutamate-induced $Ca^{2+}$ influx in postsynaptic neurons and astrocytes that in turn activate the production of vasodilators, like nitric oxide (NO) (Dreier 2011). Neurovascular coupling can be mediated by neuronal signaling mechanisms via glial pathways, or by diffusion of products of neuronal activity without the involvement of glial cells (Attwell and Iadecola 2002). Specific receptors for the vasoactive mediators released by neurons exist on BMEC, smooth muscle cells and on astrocytes. Therefore, the pericytes in the capillaries effect contraction or dilation in response to the stimuli released in their vicinity as well as through direct neuronal innervation (Hamel 2004; Iadecola 2004; Stanimirovic and Friedman 2012). In addition, after brain injury, neural growth factor (NGF) upregulates VEGF production and induces reparative neoangiogenesis and wound-healing (Lazarovici et al. 2006). In turn, BMEC nourish neighboring neurons, release trophic factors, guide developing axons, protect neurons against stress and provide a niche for supporting NPC (Guo et al. 2008; Makita et al. 2008; Ohab et al. 2006). It has also

been described that BMEC modulates the self-renewal and neurogenesis of neural stem cells (Li et al. 2006). Therefore, the mutual modulation of NPC and BMEC promotes the proliferation/migration of these cells, ultimately controlling their fate (Vissapragada et al. 2014).

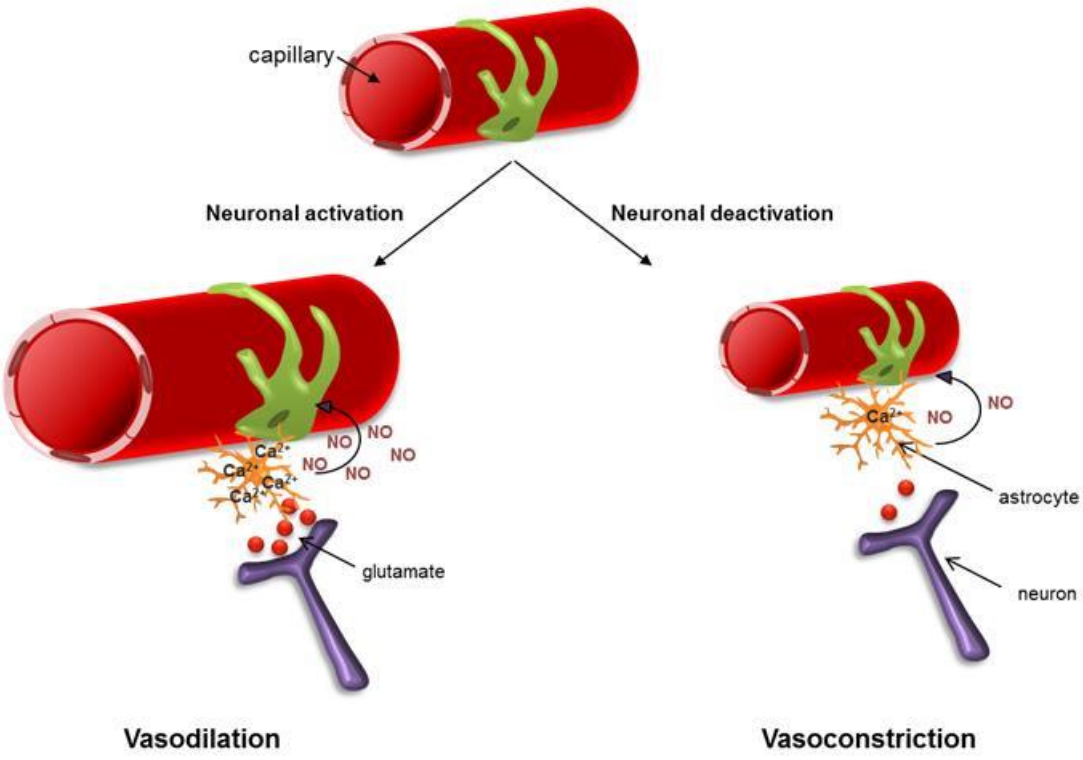


**Figure 1.8 - Schematic model of neurovascular coupling.** The release of glutamate by neurons causes elevated cytoplasmic $Ca^{2+}$ in astrocytes leading to the production of vasodilator nitric oxide (NO). NO leads the endothelium to signal the surrounding smooth muscle cells to relax, resulting in vasodilation and increasing blood flow. On the other hand, low levels of glutamate lead to vasoconstriction.

### *2.2.2. Vascular niches*

The neurovascular coupling may also be associated with neurogenesis and oligodendrogenesis. Neurogenic niches are the micro-anatomical units where neurogenesis occurs in the adult mammalian brain. In rodents, these niches are mainly located in the subventricular zone of the lateral ventricles and in the subgranular zone of the dentate gyrus in the hippocampus (Butti et al. 2014). There the progenitor cells are in close proximity to the capillaries (Goldenberg et al. 2008; Tavazoie et al. 2008) that lack astrocyte endfeet and pericyte coverage (Fig. 1.9), giving them direct access to vascular and blood-derived signals. Within this vascular niche, BMEC secret a number of diffusible signals such as VEGF, brain-derived neurotrophic factor (BDNF),

and the pigment epithelium-derived factor, as well as unidentified factors that affect neural precursors, promoting stem-cell renewal and subverting stem-cell fate decisions (Ramirez-Castillejo et al. 2006; Shen et al. 2004; Tavazoie et al. 2008). Therefore, the vascular compartment within the niche has the unique opportunity to regulate neural stem-cell behavior, which is further influenced by ependymal cells and the CSF (Tavazoie et al. 2008).

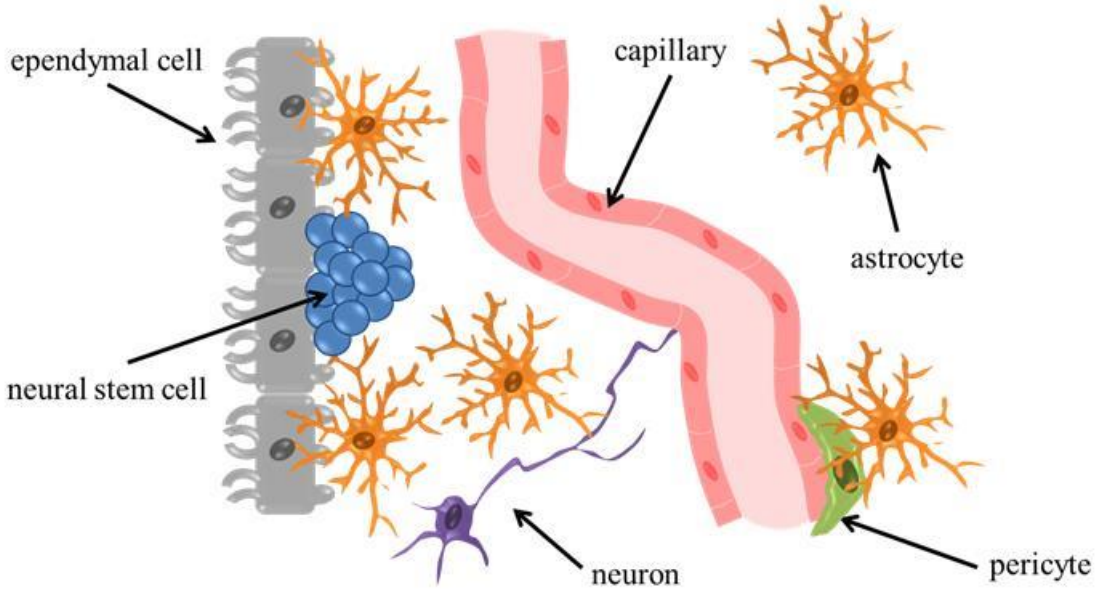


**Figure 1.9 - Stem cell niches are micro-anatomical units where neural stem cells interact intimately with ependymal cells and with capillaries, mainly within the subventricular zone.** Neural stem cells receive signals from ependymal cells and the cerebro-spinal fluid, as well as from capillaries, which lack astrocyte endfeet and pericyte coverage and therefore give them direct access to vascular and blood-derived signals. From (Cardoso et al. 2010).

BMEC also have a fundamental role in oligodendrocyte differentiation. In adverse conditions, the mature oligodendrocytes secrete high levels of MMP-9 to promote vascular remodeling (Seo et al. 2013), which as abovementioned degrades the basement membrane and may induce BBB breakdown if exacerbated (Miyamoto et al. 2014). However, the close proximity of OPC with BMEC may help the mutual remodeling and repair of these cells (Pham et al. 2012). In this oligovascular niche, BMEC may promote the switch of NPC into oligodendrocyte linage cells and stimulate the proliferation of OPCs through the release of trophic factors, such as the BDNF and the basic fibroblast growth factor (bFGF) (Arai and Lo 2009; Xing et al. 2012), as well as increase their mobility through VEGF (Hayakawa et al. 2011; Hayakawa et al. 2012b). As the neurovascular niche, the oligovascular niche may therefore provide an important mechanism for both angiogenesis and oligodendrogenesis in the adult white matter.

Lastly, pro-inflammatory and other immunological mediators can also regulate these niches by controlling several processes in neural precursors such as migration, proliferation, quiescence, cell-fate choices and survival of neural stem cells (Gonzalez-Perez et al. 2012).

### *2.2.3. Axon-myelination*

OPCs receive direct synaptic input from neurons giving rise to an axon-OPC synaptic communication. In this manner, neuron activation increases OPC proliferation, differentiation and myelination (Barateiro and Fernandes 2014; O'Rourke et al. 2014). Myelin forms a complex and dynamic process of cell-cell interaction that supports axonal integrity and survival, and can be modified by functional experience (Nualart-Marti et al. 2013). Myelination initiates in response to axon-glia recognition mediated by membrane CAMs triggering reorganization of the glial cytoskeleton and cell polarization (White and Krämer-Albers 2014). When mature, oligodendrocytes form multilayered stack of membranes tightly attached at their cytosolic and external surfaces – the myelin sheets – that wrap neuronal axons (Fig. 1.10) and speed the conduction of action potentials to provide efficient impulse propagation in large size animals (Barateiro and Fernandes 2014). Each oligodendrocyte as the capacity to myelinate oligodendrocytes myelinate up to 50 axonal segments simultaneously (White and Krämer-Albers 2014). These myelinated axon segments of nerve fibers are known as internodes and are delimited by the areas of naked axons where action potentials are generated, the nodes of Ranvier (Nualart-Marti et al. 2013). Myelin internodes have a high electrical resistance, and promote rapid conduction of the action potential from one node of Ranvier to the next. Thus, myelin enhances the integration of information across spatially distributed neural networks supporting cognitive and motor functions (O'Rourke et al. 2014). In addition, as axons are not only electrically but also metabolically isolated, oligodendrocytes provide trophic support to axons and promote their viability (Blank and Prinz 2014; Simons et al. 2014).

### *2.2.4. Astroglia-neuron crosstalk*

Astrocytes are also important for proper neuronal function and, as indicated by the enervation of brain capillaries by neurons, the interactions between these elements are essential for an efficient NVU. Below we discuss some of the main interactions between these cells.

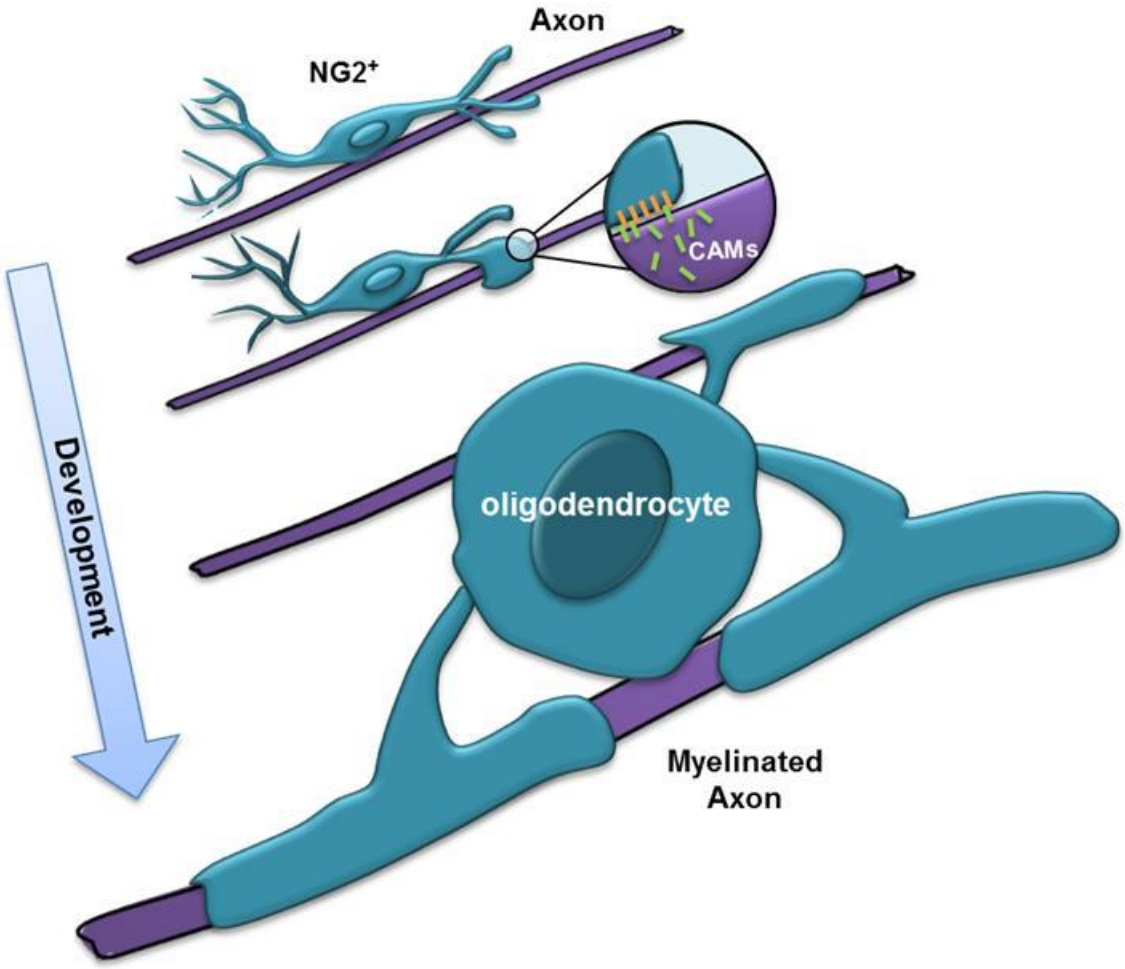


**Figure 1.10 - Development of oligodendrocytes and myelination.** Immature oligodendrocyte precursor cells that are positive for NG2 differentiate into myelinating mature oligodendrocytes. Myelin sheets connect with axons via cell adhesion molecules (CAMs). Adapted from (Lee and Fields 2009).

### *Axonal ensheathment by astrocytes*

Astrocytes are involved in axonal guidance during CNS development (Marchetti 1997). When in close contact with both axons and oligodendrocytes, astrocytes form membranous blebs (Ioannidou et al. 2014) that ensheath axons and synapses by extending thousands of thin processes, with typically only one or two in contact with blood vessels or CNS boundaries (Freeman 2010; Molofsky et al. 2012). The percentage of synapses ensheathed by astrocytic processes as well as the absolute number of synapses ensheathed by one astrocyte varies greatly with synapse type and brain region. In the cortex, astrocytic processes ensheath a substantial amount of neuronal synapses, of nearly ~90% (Magistretti and Chatton 2005). In the hippocampus ~60% of synapses are ensheathed by astrocyte processes (Ventura and Harris 1999) while in the cerebellum 67% of parallel fibers and 94% of climbing fiber synapses are connected to astrocytic extensions (Xu-Friedman et al. 2001). In addition,

there are differences between species. In rodents, one astrocyte ensheathes thousands of synapses (Bushong et al. 2002) while in the human cortex it might be more than one million (Oberheim et al. 2006). Astrocyte ensheathment is subject to dynamic modulation (Hatton 1997). Structural assembly of excitatory synapses can be mediated by physical contact with astrocytes as well as by different astrocyte-secreted proteins (Elmariah et al. 2005). When cultured in the presence of astrocytes or of astrocyte-conditioned medium, neurons can form 10 times more excitatory synapses and significantly increase synaptic functionality compared to monocultures (Ullian et al. 2001). Aside from promoting excitatory synapse formation, astrocytes can also stimulate the formation of inhibitory synapses by secreting molecules that regulate presynaptic and postsynaptic differentiation (Elmariah et al. 2005; Hughes et al. 2010).

***Regulation of neuronal activity-dependent blood-flow***

As aforementioned, astrocytes can recognize vasodilator mediators secreted by neurons, and thus play a key role in the regulation of blood flow. During neuronal activity, glutamate is released and acts *via* neuronal N-methyl-D-aspartate (NMDA) receptors to activate neuronal NO synthase (nNOS), resulting in the release of NO. This molecule acts on smooth muscle cells, increasing blood flow (Fergus and Lee 1997). In addition, glutamate released by neurons can act on astrocyte metabotropic glutamate receptors, leading to the release of vasoactive messengers from astrocytic endfeet and to simultaneous changes in vessel diameter (Takano et al. 2006; Zonta et al. 2003). This suggests that astrocytes may contribute to the neurovascular coupling, regulating cerebral blood flow by triggering either vasodilation or vasoconstriction (Howarth 2014), depending on which signaling pathway dominates.

### *2.2.5. Microglia-neuron crosstalk*

Alike the remaining glial cells, microglia are also in close communication with neurons. These brain-resident immune cells account for 10–20 % of glial cells, with similar numbers to neurons (Yao and Tsirka 2014), and showing from brain region to brain region different densities, molecular characteristics, morphologies and even responsiveness (Neiva et al. 2014). The microglia-neuron interaction is a complex process that modulates healthy neuronal functions by several processes such as glutamate uptake, removal of cell debris, production of neurotrophic factors (Fig. 1.11), as well as physical association with endangered neurons in the case of detrimental conditions (Kettenmann et al. 2011; Perego et al. 2011). Hence, healthy CNS ensures that microglial cells exert a protective role and do not develop unwanted patterns of

activation with damaging consequences for the neighboring cells (Figueiredo et al. 2008). Neurons maintain microglial resting state through physical contact or release of neurotransmitters, peptides and/or growth factors which bind to receptors on the microglial membrane (Bessis et al. 2007; Kraft and Harry 2011). In example, the physical connection of CX3CL1 (expressed by neurons) and CX3CR1 (expressed by microglia), not only keeps microglia in a resting surveillance state but it is also involved in synaptic pruning (Neiva et al. 2014), as discussed further ahead. The neuronal release of neurotrophins, such as NGF for example, controls the expression of MHC-class II in microglia (Neumann et al. 1998), whereas the neuronal secretion of CD22 inhibits microglial release of pro-inflammatory cytokines by acting on CD45 receptor (Mott et al. 2004). The physical interaction between neuronal CD200 and its receptor CD200R present on cells of myeloid origin, including macrophages and microglia, may be important for controlling tumor necrosis factor (TNF)-α released by microglia. Loss of CD200 implicates a more activated morphology along with increased expression of CD11b, CD45 and of inflammatory mediators (Broderick et al. 2002; Lyons et al. 2007). These interactions demonstrate the need of a mutual control between neuronal and microglial functions in order to maintain the integrity of CNS circuits.

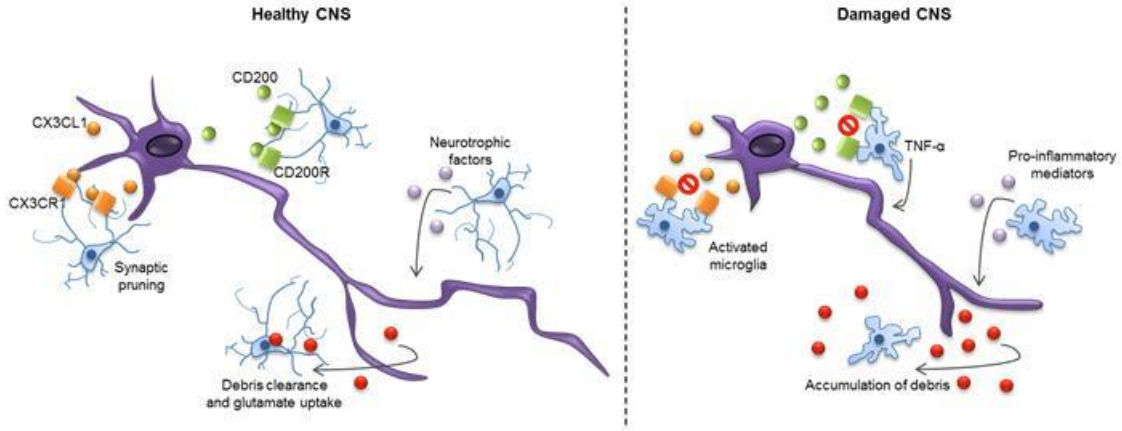


**Figure 1.11 - Microglia-neuron crosstalk in central nervous system (CNS)** in health and inflammatory conditions. In healthy CNS, fractalkine (CX3CL1) released by neurons connects with its receptors on microglial cells (CX3CR1) keeping them on a resting state. In addition, binding of the neuronal CD200 to its microglial receptor (CD200R) inhibit the production of pro-inflammatory cytokines. Also, microglia clear the debris and damaged cells, as well as excessive glutamate. However, under inflammatory conditions the bonds CX3CL1/CX3CR1 and CD200/CD200R are disturbed, leading to the activation of microglia with associated production of pro-inflammatory cytokines and accumulation of debris and glutamate in the extracellular medium.

### *2.2.6. Synaptic pruning by glia*

For normal neuronal synaptic activity, the removal of excess excitatory synapses is fundamental during embryogenesis and in the postnatal brain (Ponomarev et al. 2012; Tremblay and Majewska 2011). In the same manner, synapse pruning is equally important in the mature CNS in order to prevent epileptic activity and in the adaptive remodeling of neural circuits. Microglia are able to monitor synaptic activity and remodel specific regions of neuronal dendrites by synaptic stripping and thus limiting secondary neurodegeneration after brain injury (Kettenmann et al. 2011; Kraft and Harry 2011). Still, these cells are not alone in synaptic pruning. Recent studies have reported that astrocytes can control synapse elimination by releasing signals that activate the complement cascade (reviewed in Clarke and Barres 2013). Still, astrocytes may have a more direct role in neuronal debris and synapse elimination. Astrocytes not only express phagocytic proteins (Lööv et al. 2012; Sokolowski et al. 2011; Yu et al. 2008), but they are also able to phagocyte in response to developmental axon death (al-Ali and al-Hussain 1996), to brain injury engulfing whole dead cells and peptides (Jones et al. 2013; Lööv et al. 2012), to lipopolysaccharide (LPS) (Kalmár et al. 2001), and even in healthy CNS conditions (Nguyen et al. 2011). This shows the significance of microglial and astrocytic phagocytic activities in synapse elimination to sculpt neural circuits, regulating neuronal function from development to degeneration.

## 3. Main mechanisms of the inflammatory responses in the developing brain

The inflammatory reactions in the CNS to protect neurons from more serious damage are known as ‘neuroinflammation’. The effects of neuroinflammation on the developing brain are still a growing area in research. In the healthy developing brain inflammatory mediators play an important role in neurogenesis, apoptosis and pruning of neuronal processes. However, during pro-inflammatory conditions the developing neural circuits may be negatively affected.

### 3.1. From the periphery to the parenchyma

The first line of defense against pathogens is the local innate immune system that begins the process of tissue repair and restoration of homeostasis. Peripheral innate

immune cells include natural killer cells, natural killer T cells, mast cells, granulocytes and T cells (Amor et al. 2014). CNS-specific T cells residing at the limitants of the brain coordinate the CNS communication with the periphery while mediating their local functions. These cells are not only involved in the immune response in inflammatory scenarios but also in the maintenance of the functional plasticity of the healthy brain (Schwartz et al. 2013). In addition, the complement system of the innate immune response helps antibodies and phagocytic cells to clear pathogens. Given that it can be "recruited" by the adaptive immune response, it is considered a link between the two types of immune response. The adaptive system is directed to the infectious agent and as so, if the source is a neuron, it can contribute to neuronal damage. Overall, the extent and type of adaptive T-cell-mediated immune response is determined by the local innate response (Amor et al. 2014).

### *3.1.1. Antigen Presentation*

In the peripheral immune system, the main antigen presenting cells (APC) are the dendritic cells (DC). These are essential for antigen processing, transport to lymphoid organs, and subsequent antigen-specific activation of naïve T cells, linking innate and adaptive immunity. Differentiated DC express both CD11c and MHC-class II, which can be further induced upon activation and subsequent maturation of the cells (Olah et al. 2011). In the brain, activated microglia can express markers of APC, including MHC-class II and CD86 (David and Kroner 2011), which allow them to interact with T lymphocytes and to recognize and respond to an antigenic peptide (Bomstein et al. 2003; Butovsky et al. 2001; Schwartz et al. 2006). In addition, during inflammation BMEC are capable of presenting antigens to T cells and their expression of MHC-classes I/II and co-stimulatory molecules are upregulated by microglia-derived TNF-α (Turesson 2004; Yang et al. 2013). The release of chemoattractive factors therefore recruits and guides immune cell populations to the CNS, with presentation of antigens to T cells subsequently helping the adaptive immunity in resolving the potential source of injury (Kettenmann et al. 2011).

### *3.1.2. The blood-brain barrier as a signaling interface*

BMEC are the first cells of the CNS to contact with toxic molecules in the systemic circulation, as so they might be the first players in the signaling mechanisms that trigger neuropathology. During injury BMEC produce mediators like cytokines and NO (Brix et al. 2012; Jayakumar et al. 2012), which are secreted into either the CNS

compartment or the blood. In fact, vascular signals cause the release of cytokines by BMEC into the brain parenchyma determining the subsequent action of other brain cells, and in the same manner there are signals released from the brain parenchyma into the periphery *via* BMEC (Carvey et al. 2009; Lee and Slutsky 2010; Paolinelli et al. 2011).

During systemic inflammation, blood-borne cytokines might affect the function of the CNS by crossing the BBB for direct interaction with brain tissue. The number of cytokines that do so is considerably large, ranging from interleukin (IL)-1β, to IL-6, and to TNF-α (Quan 2008). In addition, BMEC are able to recognize and respond to microbial infection, being directly activated through the recognition of pathogen-associated molecular patterns (PAMP), like LPS, by Toll-like receptors (TLR) (Mallard et al. 2009). Hence, BMEC have cytokine binding sites, which alter intracellular function (receptors) or convey these molecules across the BBB (transporter). Given that BMEC transfer information from the blood to the CNS, the vascular system plays a key role in immune-CNS communication, responsible for activation of brain circuits and consequent CNS-mediated effects.

### *3.1.3. Recruitment and extravasation of leukocytes*

The response of BMEC to the inflammatory stimuli can induces the activation of microglia and astrocytes by itself, However, recent studies suggest that there may be a maximum number of microglia allowed to proliferate in the brain and that the proliferation of microglia depends on its local cell expansion and self-renewal (Smith et al. 2014). Therefore, there might urge in different disease processes to recruit to the CNS additional immune cells from the blood-circulation to help resolve the inflammatory response and restore homeostasis. Leukocyte recruitment occurs only under certain defined host conditions and is tightly controlled by the highly specialized BBB. Therefore, monocyte-derived macrophages are usually excluded from the healthy host brain and are recruited only to resolve an acute CNS injury (Schwartz et al. 2013). Interestingly, resting T cells are unable to cross the healthy BBB given that the endothelium limits their passage to newly activated lymphocytes (Engelhardt 2006).

The paracellular transport of myeloid cells requires chemokine receptors and other adhesion molecules. In this process there is also expression of novel surface antigens and production of mediators that build up and maintain the inflammatory response of the brain tissue (Kraft and Harry 2011). Pro-inflammatory mediators may activate BMEC leading them to increase the expression of CAM of the E-selectin subfamily (Engelhardt 2009). The chemokine CCL-2 (or monocyte chemoattractant protein-1) and

the monocytes expressing its receptor (CCR-2) are increased during inflammation, in what appears to be a direct link to the recruitment of monocytes (D'Mello et al. 2009). The binding of the BMEC selectins to the integrins on monocytes causes them to slow and roll along the luminal surface of the vessel, in a process called tethering. When reaching the paracellular junctions they transmigrate into the brain (Carvey et al. 2009) in a well-regulated process with rapid junction restoration (Wallez and Huber 2008).

In addition to paracellular migration, leukocytes may also enter the brain by transcellular migration (Carman and Springer 2004) in a process that requires the involvement of MMP to digest the basement membrane (Man et al. 2007). Mononuclear cells are able to penetrate directly through the cytoplasm of BMEC by diapedesis, which does not disrupt TJ (Engelhardt and Wolburg 2004). Even though JAM-1 is located at TJ, this protein can redistribute to the apical surface of the endothelium during inflammation or monocyte transmigration to bind to the monocytes' integrins (Ivey et al. 2009; Nourshargh et al. 2006). After the leukocytes enter the BMEC, the luminal membrane closes before the opening of the abluminal membrane in order not to create a fluid-filled channel through the cell (Carman and Springer 2008; Wallez and Huber 2008).

The majority of these recruited monocyte-derived macrophages arrive with a delay relative to the initial injury-associated breach of the BBB to facilitate the resolution of the local immune response. They do so by displaying an anti-inflammatory activity required for the regulation of the activated microglia (Schwartz et al. 2013). Still, it is important to notice that in areas of BBB disruption this process may enhance the infiltration of circulating lymphocytes and monocytes/macrophages, or even components of plasma, such as fibrinogen, that trigger microglial response (Kraft and Harry 2011).

## 3.2. Glia's response to inflammation

Microglia and astrocytes are essential for proper neuronal functioning. They quickly intercede when neurons become injured or stressed, and their pathological impairment could have disturbing consequences on brain function (Streit 2002).

### *3.2.1. Astrocytes' activation*

During inflammation, astrocytes may be targeted by pro-inflammatory cytokines or be directly infected by several pathogens, given that they express receptors that allows them to recognize various types of pathogens, like TLR2 (Combes et al. 2012). Their

activation is morphologically identified by the hypertrophy of their cellular processes, in addition to proliferation (also known as astrogliosis) and redistribution around inflammatory cells. At a molecular level there is upregulation of intermediate filament proteins, mainly of GFAP, and of neural stem cell markers vimentin and nestin (Hernandez et al. 2002; Pekny et al. 2007). These $GFAP^+$ cells control the adult neurogenesis, and may even be precursors to neurons that are added in the adult life (Pekny et al. 2007). Depleting GFAP would alter astrocytes' differentiation state into more immature cells (Emsley et al. 2004). In addition, reactive astrocytes also synthesize intercellular mediators that mediate tissue repair (Akrout et al. 2009; Pekny et al. 2014). This is the case of CX3CL1 and of high-mobility group-box-1 (HMGB1). The latter is a damage-associated molecular-pattern molecule that promotes neurovascular remodeling through endothelial progenitor cells (Hayakawa et al. 2012a). In addition, astrocytes may decrease brain inflammation by inhibiting microglia from producing IL-12, and thus blocking these cells from presenting antigens (Dietrich 2002; Dietrich et al. 2003). Reactive gliosis ranges in a scale from mild to very prominent (Fig. 1.12). The latter is connected to the formation of a glial scar, where reactive astrocytes form a physical barrier around a lesion separating it from the surrounding tissue [for further reading, (Raposo and Schwartz 2014)], and that has been reported in several models for degenerative and inflammatory disorders (Bao et al. 2012; Grigorian et al. 2011; Wu et al. 2012). However, astrocytes might also produce several pro-inflammatory molecules which ultimately increase permeability, promote lymphocyte trafficking and could further damage the CNS (Hansson and Rönnbäck 2003; Langford and Masliah 2001). Nonetheless, astrocytic activation is in its majority a neuroprotective attempt to supports CNS regeneration.

### *3.2.2. Microglia's response to inflammation*

As abovementioned, microglia are the principal immune cells of the CNS, surveying local microenvironment and changing the phenotype in response to homeostatic disturbances (Ransohoff and Perry 2009). These disturbances may be due to release of "danger factors" such as HMGB1, chemokines CCL2 and CCL21, among others (Kraft and Harry 2011), or due to the loss of stimulation of microglial membrane by ligands such as CD200, CX3CL1, neurotransmitters and neurotrophins (Biber et al. 2007; Pocock and Kettenmann 2007). As abovementioned, CX3CL1 induces a resting state on $CX3CR1^+$ microglia. The interruption of this complex allows microglial activation and enhanced response to activating signals. Once activated, microglia perform several macrophage-like functions including phagocytosis, remove

cellular debris, inflammatory and anti-inflammatory cytokine production, antigen presentation and even promote remyelination (Garden and Möller 2006). Though their activation may trigger neurotoxic pathways, as being part of the innate immune system, they are interference is indeed crucial in controlling inflammation and in the mediation of neuroprotection and repair (Amor et al. 2014; Olah et al. 2012).

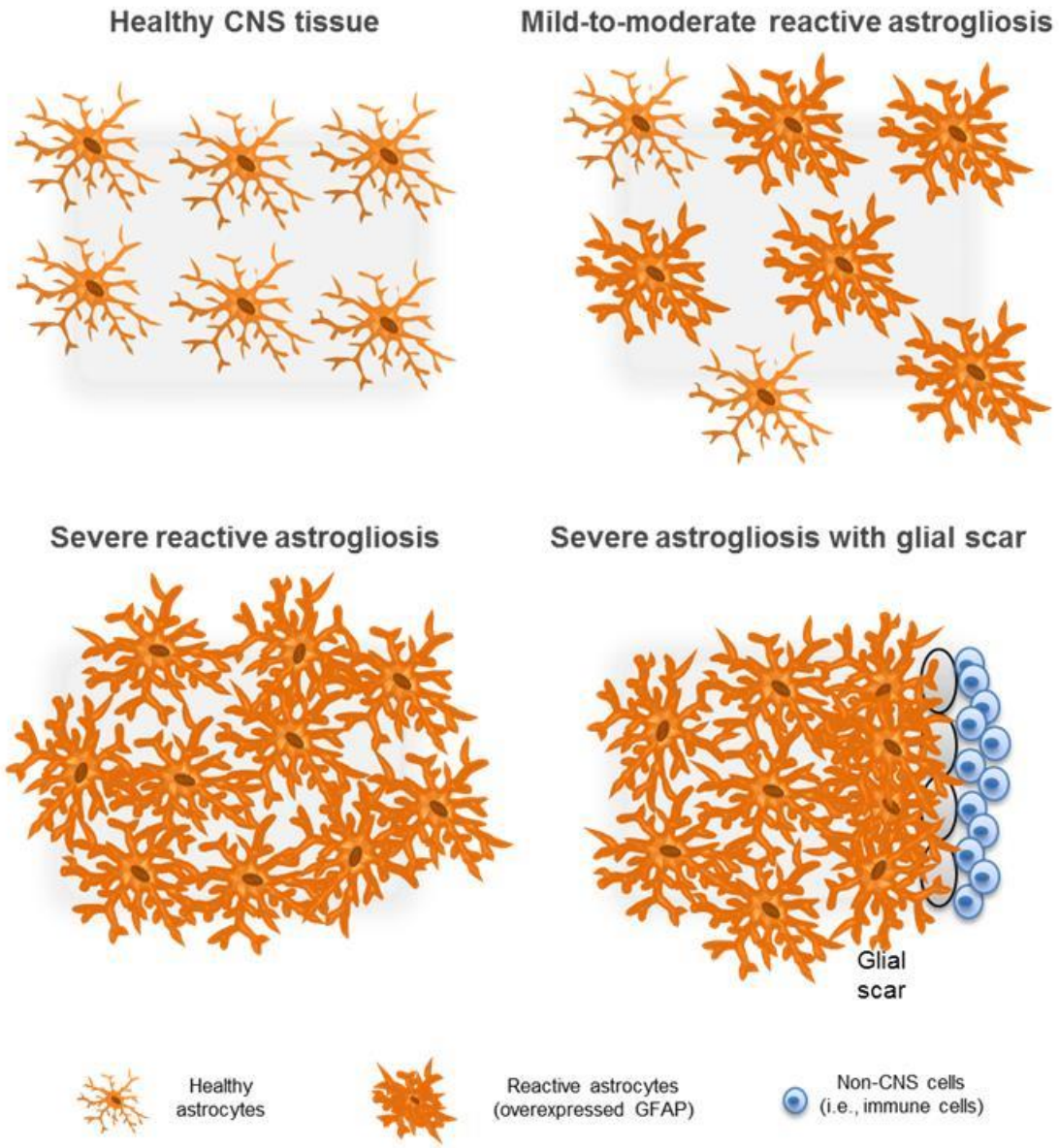


**Figure 1.12 - Schematic representation of the different stages of reactive astrogliosis.** In the healthy central nervous system (CNS) astrocytes have no-overlapping domains. The individual domains are preserved in mild to moderate reactive astrogliosis, though most cells display hypertrophy and express high levels of GFAP. The proliferation of $GFAP^+$ astrocytes is further promoted in severe reactive astrogliosis, with additional overlap of astrocytic domains. Finally, there may be glial scaring with astrocytes bordering the damaged regions with recruitment of several immune cells. Adapted from (Sofroniew and Vinters 2010).

***Types of microglia***

Microglia can shift activity states depending on surrounding conditions. Under normal conditions they are characterized by a small cell body with fine, ramified processes and low expression of surface antigens. In this “resting” state, microglial cellular processes are in surveillance mode, being constantly in motion to monitor their environment for signals from the surrounding cells. Upon brain injury, microglia rapidly proliferate and transform their morphology from long to short processes, the “active” state, also releasing proinflammatory molecules and increasing the expression of immunomodulatory surface antigens (Kettenmann et al. 2011; Weinstein et al. 2010).

Microglia’s “resting” is not a non-active state. As upcited, these cells are constantly monitoring the normal CNS. Not only neurons but also astrocytes appear to take part in the maintenance of this state given that they too express CX3CL1, and their conditioned medium is capable of increasing ramification of blood monocytes in culture (Wollmer et al. 2001). Other molecules secreted by astrocytes can induce this state, as is the case of TGF-β, macrophage-colony stimulating factor (M-CSF), and granulocyte/macrophage-colony stimulating factor (GM-CSF) (Schilling et al. 2001).

Microglial activation is increasingly recognised as an early sign of CNS inflammation. As part of the innate immunologic system, microglia recognize the injury or pathogen and quickly initiates production of pro-inflammatory molecules (Abbott et al. 2010). Circulating pro-inflammatory cytokines like IL-1, IL-6 and TNF-α are major activators of microglial cells, *via* a cyclooxygenase (COX)2-dependent mechanism that subsequently produces prostaglandin E2 and may induce fever. Blood-borne proteins such as thrombin, plasminogen activator, and the complement system have also been shown to activate microglia (Ryu et al. 2000; Siao and Tsirka 2002; Webster et al. 2000). Activated microglia are able to recruit the appropriate cells to clear debris, and minimize primary neuronal damage and the secondary wave of neuronal death (Turrin and Rivest 2006).

Microglia’s morphological responses differ depending on the type of insult. Soluble signals from endangered neurons and astrocytes, as well as plasma-derived factors, induce microglia’s proliferation and transform their morphology to bushy, to hypertrophic or to rod shapes with reduced cellular processes. These morphological forms in microglial activation may interchange during the progression from the ramified to the amoeboid macrophage-like appearance (Karperien et al. 2013; Kettenmann et al. 2011; Perego et al. 2011; Weinstein et al. 2010). Local densities of microglia can also increase by proliferation, to provide more cells for both defense against the stimuli and for protection and restoration of tissue homeostasis. When not at rest, microglia display altered expressions of inflammatory cytokines, induction of surface molecules

for cell-cell and cell-matrix interactions. All of these aim to recognize possible complement proteins deposited on the aberrant synapses or neuritis that need to be removed (Rezaie et al. 2002; Stence et al. 2001).

Microglial activation does not imply that all cells are activated, and in fact more than one functional state can coexist. As upcited, there is regional heterogeneity in the microglia population. A reason to such heterogeneity may be due to differences in the expression of multiple proteins by microglia of different CNS regions, and from white to gray matter. Neurotrophins, for example, were seen in microglia that not only showed a region-specific pattern but also differed within the regions themselves, being produced by certain subpopulations (Schmid et al. 2009). Interestingly, this profile is paralleled by heterogeneity of oligodendroglial subpopulations in subregions of gray and white matter (de Haas et al. 2008; Kitada and Rowitch 2006).

Following defense activation, microglia may assume reparation functions and eventually reverse to a resting state or remain primed. Post-activated microglia may remain morphologically undistinguishable from the unactivated cells, though having long-lasting functional adjustments Studies on microglial activation after stroke indicate that long-term activation of microglia in the subventricular zone is important for regulating long-lasting neurogenesis in this area (Kettenmann et al. 2011; Thored et al. 2006).

***Microglial phenotypes***

Several reports have discussed the existence of two distinct sub-populations of activated microglia in the normal mouse brain: type M1 and type M2 (Fig. 1.13). Both types have similar phagocytic activity, but they show distinct phenotypes such as cell-surface markers, mRNA expression, and growth factor dependency. In fact, one has a pro-inflammatory phenotype (classical activation) associated with demyelination, while the other displays an anti-inflammatory and reparative phenotype (alternative activation) responsible for remyelination (Durafourt et al. 2012; Lacy-Hulbert and Moore 2006; Olah et al. 2012).

M1 microglia can be triggered in response to GM-CSF, LPS, as well as pro-inflammatory cytokines interferon (IFN)-γ and TNF-α (Durafourt et al. 2012). This type is characterized by increased chemotaxis, production of inflammation mediators and cytokines, and they have high capacity to present antigen mediating both innate and adaptive responses (Gabrusiewicz et al. 2011). They upregulate the expression of several receptors such as TLR2/4 (for pathogen recognition), Fc (to bind antibodies), CCR7 (of which the ligand is CCL21) and of CD80 (for T cells activation) (Durafourt et al. 2012). M1 also has high superoxide production with consequent toxic effects and

cell death of cholinergic and dopaminergic neurons (Sawada 2009). In addition, the classical activation phenotype includes production of NO, reactive oxygen species (ROS), TNF-α and IL-1β, IL-6 and IL-12 (Butovsky et al. 2005; Perego et al. 2011; Voisin et al. 2010). Still, a report by Sawada and collaborators demonstrate that even though microglia activated by LPS and IFN-γ may be neurotoxic in aged mice, in the neonatal brain it can actually be neuroprotective (Sawada 2009).

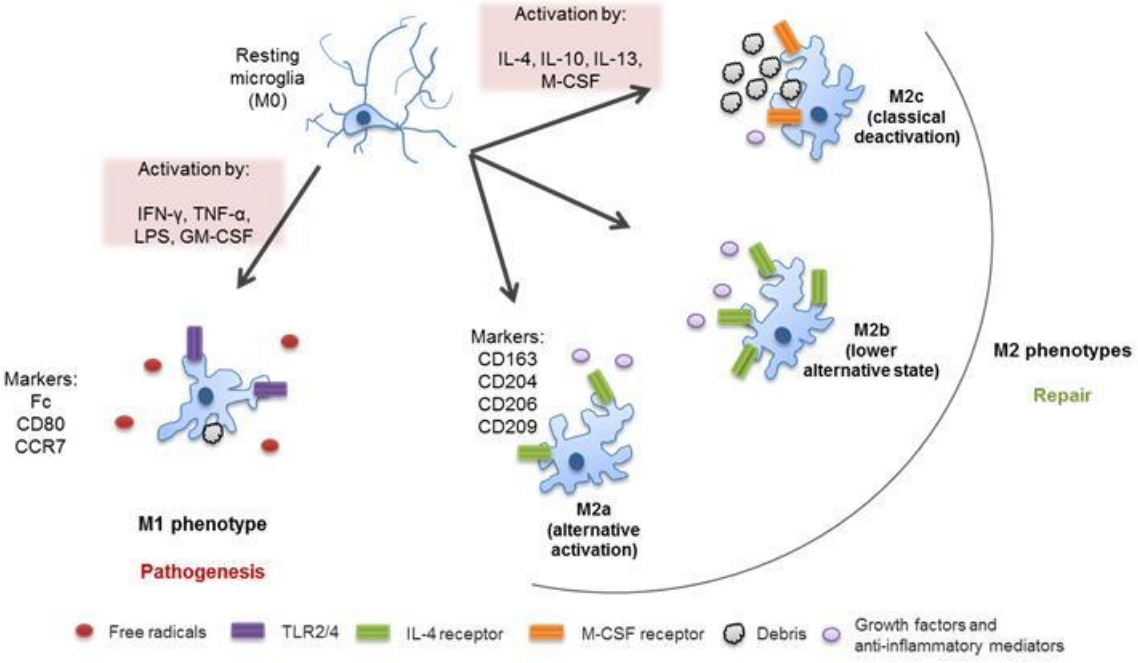


**Figure 1.13 - Induction of microglia phenotypes.** A pro-inflammatory immune cell M1 phenotype is acquired upon the classical activation of resting microglia (M0) by interferon (IFN)-γ, tumor necrosis factor (TNF)-α, granulocyte macrophage colony-stimulating factor (GM-CSF) or lipopolysaccharide (LPS). The binding LPS to Toll-like receptors triggers the release of pro-inflammatory cytokines and free radicals, thus amplifying the neuroinflammatory response. On the other hand, interleukin (IL)-4, IL-10, IL-13 or macrophage colony-stimulating factor (M-CSF) induce an anti-inflammatory M2 phenotype representing a beneficial activation state promoting repair that scavenges debris and releases anti-inflammatory mediators. According to the stimuli, there may be an alternative classic activation (M2a), a lower activation sate (M2b) or a classical deactivation (M2c).The activation of IL-4 receptor promotes the activation of phenotypes M2a and M2c, whereas the activation of M-CSF receptor favors the activation of M2c.

The “alternative” M2 phenotype is an apparently beneficial activation state, more related to a fine tuning of inflammation, scavaging of debris and promotion of angiogenesis (Perego et al. 2011). Is has been proposed that microglia in the normal CNS exhibit a phenotype of M2-like cells (Ponomarev et al. 2012). This phenotype can be triggered by M-CSF, IL-4, IL-10 and IL-13 (Durafourt et al. 2012). According with the

type of polarization distinct M2 populations can be differentiated, called M2a, M2b, and M2c, which differ in some cell surface properties and capacity to secrete immune mediators (Leidi et al. 2009). IL-4 promotes an alternative activation state or M2a, whereas IL-10 promotes a classical deactivation profile or M2c. Both M2a and M2c phenotypes reduce pro-inflammatory mediators. As for M2b activation profile, it shows expression of both M1 and M2c markers (Fenn et al. 2012; Mantovani et al. 2004; Mosser and Edwards 2008). Interestingly, experiments demonstrated that LPS can induce a M2b phenotype, with significantly upregulated expression of IL-4 receptor, which is re-directed towards a less inflammatory M2a profile following IL-4 addition (Fenn et al. 2012). In general, M2 phenotype is characterized by the expression of the scavengers receptors CD163 and CD204, the mannose receptors CD206 and CD209, as wells as the secretion of collagen (Durafourt et al. 2012; Gabrusiewicz et al. 2011; Voisin et al. 2010). The overexpression of M-CSF receptor (M-CSFR) expressed in activated microglial cells stimulates a 2- to 3-fold greater phagocytic capacity compared with GM-CSF-induced cells (Leidi et al. 2009). Unlike M1, the M2 type has low superoxide production with a protective effect on cholinergic neurons (Sawada 2009). It can also promote remodeling and tissue repair by releasing growth factors and modulating the immune response (Schwartz 2010). Activated microglia alter gene expression patterns towards protective and anti-inflammatory mediators such as IL-10, insulin-like growth factor (IGF)-1, TGF-β, BDNF and glial cell line-derived neurotrophic factor (GDNF), inducing neurotrophin-dependent protective activity in damaged neurons (Butovsky et al. 2005; Sawada 2009; Weinstein et al. 2010).

The M1 profile is transient with microglia returning to a surveying state. Recent studies demonstrate that IFN-γ and LPS stimulation promotes the M1 phenotype by targeting M2-associated genes, downregulating the IL-13 receptor (IL13Rα1) (Louafi et al. 2010; Martinez-Nunez et al. 2011). Its regulation by several anti-inflammatory mediators is fundamental to attenuate microglial activation and promote the anti-inflammatory M2 profile (Fenn et al. 2012). There are studies advocating that two-phenotypes is a simplification and at least one additional activation state exists, as it is for T cells (Olah et al. 2012).

***Motility and migration***

Several steps and intermediate stages can be identified in microglia, including characteristics of process withdrawal, transition or hyper-ramification, and subsequent formation of new protrusions along with motility and migration (Kettenmann et al. 2011). In the motility movement, resting microglial cells actively scan their environment with motile processes in highly random directions. By moving the fine processes

scanning can occur without cellular migration and therefore maintaining the proper functioning of the neuronal system (Davalos et al. 2005; Nimmerjahn et al. 2005). Still, the entire cell can migrate within brain tissue including translocation of their soma.

The motility of microglia is controlled by autotaxin (ATX), a phospholipase D, expressed during the CNS development and in the normal adult brain. It converts lysophosphatidylcholine into lysophosphatidic acid (LPA), which is the true molecule that cells need to migrate (Liu et al. 2009). During normal neurodevelopment, ATX and LPA are involved in the differentiation of oligodendrocyte precursor cells (Yuelling and Fuss 2008). However, under pathological conditions like LPS administration, ATX is upregulated not only in microglia (Awada et al. 2014) but also in reactive astrocytes (Savaskan et al. 2007), allowing microglia to actively move to a lesion or site of infection following chemotactic gradients. There are a number of receptor systems that control the migration of microglial cells. Filopodia protrusion and dynamic rebuilding are needed for motility and directed migration (Kettenmann et al. 2011). However, rapid morphologic responses are diminished in neurodegenerative disorders and in the aged brain, where these cells show less motility and fewer processes, supporting the hypothesis of an impaired microglial functionality (Damani et al. 2011; Meyer-Luehmann et al. 2008). Interestingly, ATX has the ability to minimize microglia cell death induced by oxidative stress suggesting a potential beneficial role for ATX (Awada et al. 2012; Awada et al. 2014).

***Phagocytosis by microglia***

Appearance of microglial cells with lipid- or myelin-loaded organelles in brain tissue sections points to phagocytic activity. The accumulation of cellular debris due to inadequate removal of dead and damaged cells may be detrimental to neighboring cells, and eventually lead to early onset of degenerative disorders and premature cell aging. Hence, the mechanism of clearance by phagocytosis is crucial in normal brain development and function, as well as in pathology and following regeneration (Schwartz et al. 2013). Microglia play a fundamental role by phagocyting apoptotic cells during development, and by pruning synapses in the postnatal brain (Kettenmann et al. 2011). In addition, they may also phagocyte molecules and debris such as amyloid-β deposits. The activation of the receptor for advanced glycation end-products (RAGE), a cell surface receptor present in microglia and other CNS cells, that also recognizes HMGB1 for example, contributes to the clearance of this exceeding molecule that could ultimately lead to Alzheirmeir's disease (Kraft and Harry 2011). Other studies also suggest that phagocytosis of injured tissue is important for remodeling and may limit secondary damage after brain injury (Zhao et al. 2007). During primary demyelination,

for example, microglial capacity to present antigen or to activate T cells are considerably low. However, during the following remyelination the accumulation and phagocytic activity of microglia is markedly downregulated. Clearance of debris by microglia may thus create an environment that is beneficial for regeneration (Voß et al. 2012).

Microglia and astrocytes express “scavenger receptors” that regulate the uptake of several substrates (Husemann et al. 2002). Their expression allows microglia to interact with astrocytes and neurons, and properly respond to their sent signals (Färber et al. 2005). GDNF and M-CSF are known to increase the phagocytic capability of the microglia (Chang et al. 2006; Mitrasinovic and Murphy 2003), whereas the prostanoid receptor subtype 2 (EP2) downregulates phagocytosis (Liang et al. 2005; Shie et al. 2005). The expression of purinergic receptors may control microglial phagocytosis, given that the activation of P2Y6 receptors are associated with the induction of activated microglia and the triggering of phagocytosis, whereas activation of P2X7 receptors suppresses phagocytosis (Fang et al. 2009; Inoue et al. 2009; Koizumi et al. 2007). The differential expression of these receptors in response to the intensity of tissue injury, the type of injurious stimuli, or the presence of other soluble signals may control the degree of microglial response (Kraft and Harry 2011).

## 4. Common neonatal clinical conditions

Human newborns, especially preterm, are at high risk of brain damage (Brochu et al. 2011). In postnatal rats, a stage of brain development equivalent to 22-28 weeks gestation in humans, the BBB is still not fully mature and the blood vessels present leakage of plasma proteins (Saunders et al. 2012). Thus, neuroinflammation during brain development may alter brain functions profoundly, contributing to lasting cognitive deficits, changes in neurogenesis and to different behavioral phenotypes and psychiatric disorders in adulthood (Conrad et al. 2014; Drouin-Ouellet et al. 2011; Elmore et al. 2014a; Green and Nolan 2014; Malaeb et al. 2014). Among the most common neonatal clinical conditions are unconjugated hyperbilirubinemia and sepsis, which have been shown to deteriorate cognitive ability as well as to increase the risk of developing dementia, cerebral palsy, among others (Przekop and Sanger 2011; Widmann and Heneka 2014). The dose and timing of neonatal insult and offspring age now emerge as important factors for evaluating neuropsychiatric disorders in adults who experienced early life infection (Majidi Zolbanin et al. 2014).

## 4.1. Neonatal jaundice

Physiological jaundice of the neonate is characterized by a preferential deposition of unconjugated bilirubin (UCB) in brain regions such as the basal ganglia, the hippocampus and the cerebellum, giving a yellowish color to the tissue (Brites 2012; Watchko 2006). Even though this condition is usually benign and UCB levels return to normal at the end of the first week of life, still there may be bilirubin-induced neurologic dysfunction with consequent minor brain deficits or with more severe encephalopathy as is the case of kernicterus that might even cause death (Shapiro 2010). UCB encephalopathy affects 0.5-2.4 children per 100,000 live births in North America and Europe (Burgos et al. 2012) and therefore it is important to understand the "how" behind such brain dysfunctions.

### *4.1.1. Bilirubin metabolism in newborns*

UCB is a tetrapyrrolic molecule and the main product of the catabolism of heme, which is mostly present in erythrocyte hemoglobin. UCB has low aqueous solubility due to its structure, requiring albumin as a carrier protein to transport it in the blood. After birth there is a massive destruction of erythrocytes, due to their shorter lifespan, favoring the accumulation of UCB in the serum (Stevenson et al. 2011). In addition, UCB clearance in the newborn no longer occurs by the maternal machinery and has therefore to be done by its own less effective UDP-glucuronosyl transferase. This enzyme is responsible for converting molecules into more soluble forms to be eliminated from the body by the kidneys. Therefore, the immaturity of this enzyme further favors UCB accumulation (Dennery et al. 2001; Gourley 1997; Kawade and Onishi 1981). In the same manner, there is increased intestinal absorption of UCB due to the presence of intestinal β-glucuronidase (an enzyme that deconjugates the conjugated bilirubin species) combined with poor intestinal flora that usually metabolizes UCB into excretable products (Takimoto and Matsuda 1971; Vitek et al. 2000). Breast-feeding is believed to increase the concentration of β-glucuronidase, promoting the reabsorption of UCB and consequent enterohepatic circulation (Gourley 1997). A small percentage of UCB also circulates in the blood unbound to albumin, named free bilirubin (Bf). This form can easily bind to phospholipid membranes or even diffuse through them (Brito et al. 2006; Stevenson et al. 2011). The low serum levels of UCB in normal infants may have a protective effect against oxidative stress injury. However, these same levels are considered to be high and possibly hazardous in preterm infants and even in normal adults (Gkoltsiou et al. 2008; Stevenson et al. 2001;

Watchko and Maisels 2003). Therefore, and given its high incidence, it is extremely important to understand the impact of hyperbilirubinemia on brain development.

### *4.1.2. Bilirubin's neurotoxicity*

During neonatal hyperbilirubinemia, BBB disturbances may facilitate the entrance of Bf into the brain parenchyma (Palmela et al. 2012), which would consequently damage the remaining cells of the CNS (Fig. 1.14). BMEC are the first brain cells to have direct contact with circulating UCB, either in its free form or bound to albumin. It has been shown that UCB impairs the cellular membrane structure, properties and function (Brito et al. 2004; Brito et al. 2001). Thus, even though the brain endothelium was long viewed as a site for UCB crossing (Katoh-Semba and Kashiwamata 1980), it is now known that this process is at the expense of compromising the endothelial barrier (Palmela et al. 2012).

BMEC and neurons are particularly sensitive to this molecule, and their contact can ultimately lead to cell death by both apoptosis and necrosis (Akin et al. 2002; Falcão et al. 2005; Falcão et al. 2007; Palmela et al. 2011). In addition, UCB triggers the release of pro-inflammatory cytokines by BMEC (Palmela et al. 2011) and glia (Fernandes et al. 2006; Fernandes et al. 2004; Silva et al. 2010), as well as to oxidative stress in both vascular and nerve cells (Brito et al. 2008a; Brito et al. 2008b; Palmela et al. 2011).

Transporters are also affected by UCB, especially in BMEC. An increased expression and plasma membrane localization of GLUT1 (Cohen et al. 2006) translates into high glucose levels inside these cells. This process is then deeply connected with UCB toxicity by further producing the upcited oxidative stress (Kapitulnik et al. 2012). In addition, efflux transporters are also altered with an *in vivo* significant basolateral to apical active efflux of this molecule (Sequeira et al. 2007) that is in agreement with the increased expression of P-gp in brain samples from a kernicterus case (Brito et al. 2013). Interestingly, P-gp expression in the rat vasculature increases with animal age, reaching its maximum level in adulthood. Hence the results in the kernicterus case may be a protective mechanism to overcome the possible immaturity of neonatal blood vessels to efflux UCB from the brain parenchyma, compared to the adult animals (Gazzin et al. 2011; Gazzin et al. 2008).

The interaction of UCB with brain cells has been shown to disassemble the cytoskeleton of neurons (Silva et al. 2002), which may also be involved in the impairment of the TJ and AJ seen in BMEC (Palmela et al. 2012). In the particular case

of BMEC, the disruption of the endothelial monolayer leads to increased permeability that has been shown to be connected with angiogenesis (Pipili-Synetos et al. 1993; Rigau et al. 2007; Wei et al. 2003). Indeed, brain samples from an autopsy material of a kernicterus case showed more vessels, though with a poorly defined lumen (Brito et al. 2012), which is associated with immature vessels alike for P-gp.

At last, UCB toxicity is accompanied by neuronal dysfunction, along with impairment of oligodendrogenesis, of myelination, and of the dendritic arborization in the cerebellum (Barateiro et al. 2013; Barateiro et al. 2012; Bortolussi et al. 2012). Moreover, other studies with kernicterus samples have shown neuronal dysfunction in different brain areas, particularly in the basal ganglia (Hachiya and Hayashi 2008). Altogether, UCB is able to trigger an injurious response in the neonatal BBB and neighboring cells.

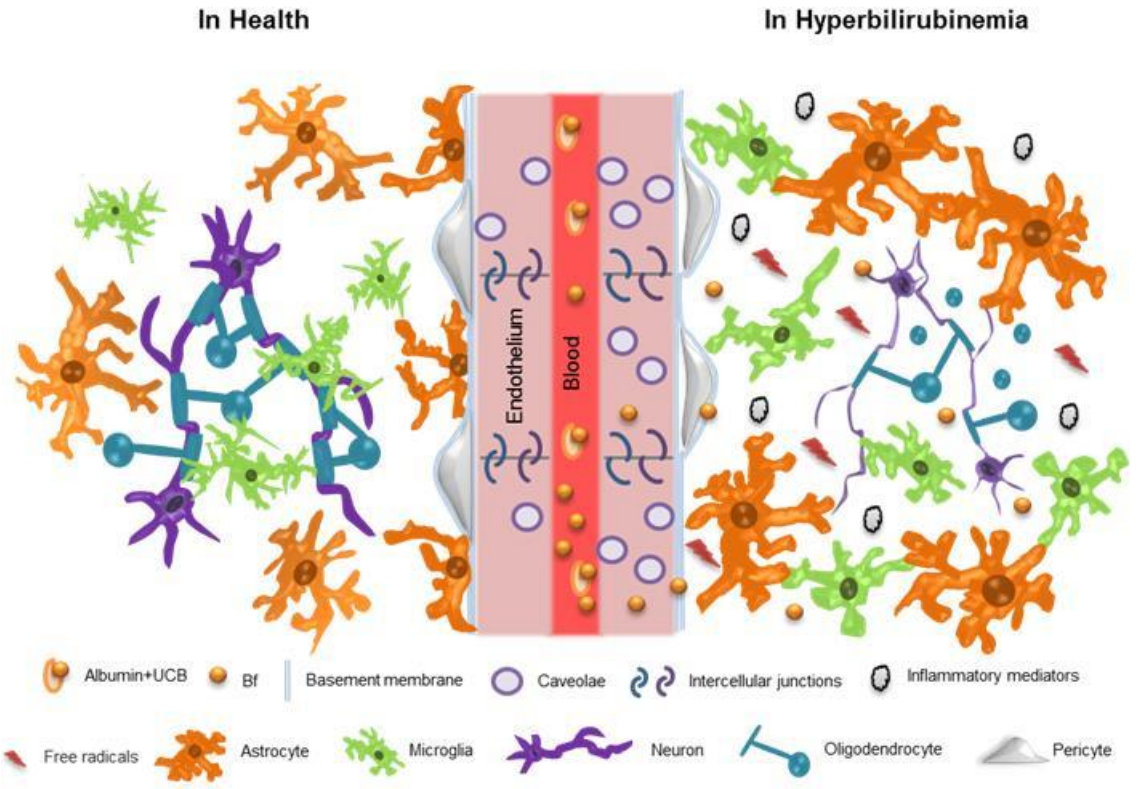


**Figure 1.14 - Main alterations occurring at the neurovascular unit during hyperbilirubinemia.** Unconjugated bilirubin (UCB) circulates in the blood bound to albumin, however when in high levels the interaction of free UCB (Bf) with the endothelium is enhanced leading to a greater passage across it into the parenchyma. Entrance is facilitated by increased numbers of caveolae and disruption of the intercellular junctions. The production of pro-inflammatory mediators and free radicals further induce damaging effects in pericytes, as well as astrocytes' and microglia reactivity, neurodegeneration and decreased number of mature myelinating oligodendrocytes.

## 4.2. Neonatal sepsis

Sepsis is a whole-body inflammatory response to a severe bloodstream infection. In past years its incidence has increased by 70% up to 37.7 hospitalization cases per 10,000 population, of which 17% ended in death (Hall et al. 2011). In the children younger than 5 years that percentage increases to 68%. Human newborns, especially preterm, are at high risk (Brochu et al. 2011) and, indeed, the neonatal period (0-27 days) accounts for 41% of deaths, particularly due to preterm births by sepsis (Black et al. 2010; Kissoon et al. 2011). The system's attempt to eliminate the pathogens may increase by itself the permeability of the BBB, induce severe brain damage and ultimately lead to sepsis-associated encephalopathy (SAE). SAE is characterized by acute to lasting deficits in cognitive functions that may become permanent even after the patient's recovery (Widmann and Heneka 2014), mostly associated with neuronal and astroglial dysfunction (Semmler et al. 2008; Stocchetti 2005).

### *4.2.1. Endotoxin recognition*

LPS is an endotoxin from the wall of gram negative bacteria that is recognized by the innate immune system, hence being widely used in both *in vitro* and *in vivo* models to mimic sepsis. Interestingly, LPS appears not to cross the BBB given that its concentration does not increase in the brain parenchyma after administration (Banks and Erickson 2010). So, its neurologic effects appear to result from a direct contact with BMEC and consequent activation of a number of signaling pathways that lead to the release of inflammatory mediators (Berg et al. 2011; Grab et al. 2011; Tripathi et al. 2009). As upcited, microglia and BMEC express TLRs that interact and respond to various PAMPs. In the case of LPS, its interaction with BMEC is through TLR4 in a process that requires the accessory proteins CD14 and myeloid differentiation 2 (MD2) (Shimazu et al. 1999; Ulevitch and Tobias 1995). The cytoplasmic protein regions of TLR4 interacts with the signaling adaptor myeloid differentiation factor 88 (MyD88) that transduces the signal to IL-1 receptor-associated kinase (IRAK)-1 and IRAK-2, resulting in transcriptional responses mediated primarily by the protein complex that controls the transcription of DNA, NF-κB, the extracellular-signal-regulated kinase (ERK), stress-activated protein kinases such as c-Jun N-terminal kinases (JNK) and p38. At last, it leads to the synthesis and release of pro-inflammatory mediators including cytokines, chemokines and IFN, as well as the upregulation of CAM (Armant and Fenton 2002; Karikó et al. 2004; Mallard et al. 2009; Spencer et al. 2011). In addition, there can be activation of COX-2 in BMEC that, as upcited, can culminate in a

fever response (Karikó et al. 2004; Kis et al. 2006; Spencer et al. 2011). In fact, administration of LPS is known to induce sickness behavior in mice, including weight loss (Lawson et al. 2013; Walker et al. 2013). COX-2 is also expressed by neurons, however, they do not influence the inflammatory response (Aid et al. 2010). All these mediators help in the clearance of bacteria, but at the same time also impair CNS functions that may induce brain damage (Nayak et al. 2014).

### *4.2.2. Sepsis neurotoxicity*

The recognition of circulation leads to a cascade of events that ultimately alter the normal functioning of the CNS (Fig.1.15). One of the first events triggered by LPS on BMEC is the upregulation of the vascular CAM (VCAM-1), a protein that mediates the adhesion of immune cells to the vascular endothelium, and it also plays a part in leukocyte-endothelial cell signal transduction (Norman et al. 2008; Ramirez et al. 2012; Ruiz-Valdepeñas et al. 2011; Steiner et al. 2010). This is simultaneous with the increase in chemokines CCL2 and CCL3, where CCL2 establishes a chemotactic gradient for the directional cell migration into the brain parenchyma, and CCL3 is responsible for the initial recruitment and activation of immune cells (Chui and Dorovini-Zis 2010). Also involved in the macrophage recruitment induced by LPS is the release of GM-CSF from BMEC (Dohgu et al. 2011), which in turn stimulates the production of granulocytes and monocytes (Erickson and Banks 2011). Surprisingly, a recent study affirms that acute neonatal LPS administration does not trigger peripheral monocyte recruitment (Yao et al. 2013). The compromise of integrity of the barrier properties by LPS stimulation, on the other hand, has a general consensus. Indeed endocytosis is augmented, with a significant increase in the area and number of endocytic vesicles (Defazio et al. 1997) (Banks and Erickson 2010; Banks et al. 1999; Defazio et al. 1997) and an impaired P-gp efflux transporter function (Salkeni et al. 2009). Moreover, TEER decreases following LPS treatment with a simultaneous increase in transendothelial permeability to the well-established tracers Evan's Blue-bound to albumin (EBA) and sodium fluorescein (NaF) (Nishioku et al. 2009; Ramirez et al. 2012; Ruiz-Valdepeñas et al. 2011). The reason behind this could be directly related to the fragmentation and low expression of TJ and AJ proteins in BMEC (Li et al. 2012; Stolp et al. 2011; Veszelka et al. 2007).

Direct effects on BMEC can also be exacerbated *via* the response of other cell types. Even though LPS does not cross a normal functioning BBB, not all brain microvessels sustain barrier properties. In fact, neurons and glial cells may respond

directly to LPS in circumventricular organs (Ott et al. 2010). As previously mentioned, astrocytes play an important role in the induction of BBB functions and in the modulation of the BBB phenotype without needing to be directly involved in the physical BBB properties (Colgan et al. 2008; Hayashi et al. 1997; Siddharthan et al. 2007). Nonetheless, exposure of BMEC to conditioned medium from LPS-treated astrocytes has been reported to aggravate BBB disruption (Landoni et al. 2012). In fact, astrocytes exposed to LPS release high levels of pro-inflammatory cytokines, particularly by immature cells (Falcão et al. 2005), directly influencing neurodegeneration (Falcão et al. 2014). In addition, *in vivo* prenatal exposure to LPS triggers extensive astrogliosis in the white matter with consequent inhibition of oligodendrocyte maturation (van den Heuij et al. 2014). Interestingly, BBB disruption under inflammatory conditions has also been verified in co-cultures of BMEC with other CNS cells, as is the case of pericytes (Veszelka et al. 2007) and of microglia (Kacimi et al. 2011; Sumi et al. 2010). This phenomenon could be associated with augmented production of vasodilator NO by microglia, pericytes and BMEC (Kacimi et al. 2011; Kovac et al. 2011), showing the susceptibility of these cells to LPS. The *in vivo* administration of LPS is during the postnatal period also activates microglia in the white matter (Pang et al. 2010), with potential long-lasting defects in oligodendroglial maturation and myelination (Favrais et al. 2011; Volpe 2011). Moreover, *in vivo* administration of LPS also has a long-term impact in the production of hippocampal neurons, displaying less synaptic contacts as well as decreased dendritic volume and complexity (Valero et al. 2014). Equally to other inflammatory conditions, LPS also induces microglia to release pro-inflammatory cytokines that mediate alterations in neuronal network activity and apoptosis (Nimmervoll et al. 2013), as well as other inflammatory mediators like HMGB1 (Lamar et al. 2011), MMP-9 and -2 (Konnecke and Bechmann 2013) and ATX (Awada et al. 2012).

Even though in the particular case of sepsis gender appears not to influence LPS-response (Pang et al. 2010), still there are some discrepancies in the inflammatory response depending on the age, concentration, single or multiple injections, time of exposure, for what we believe it is important to understand the immune reasons behind such differences.

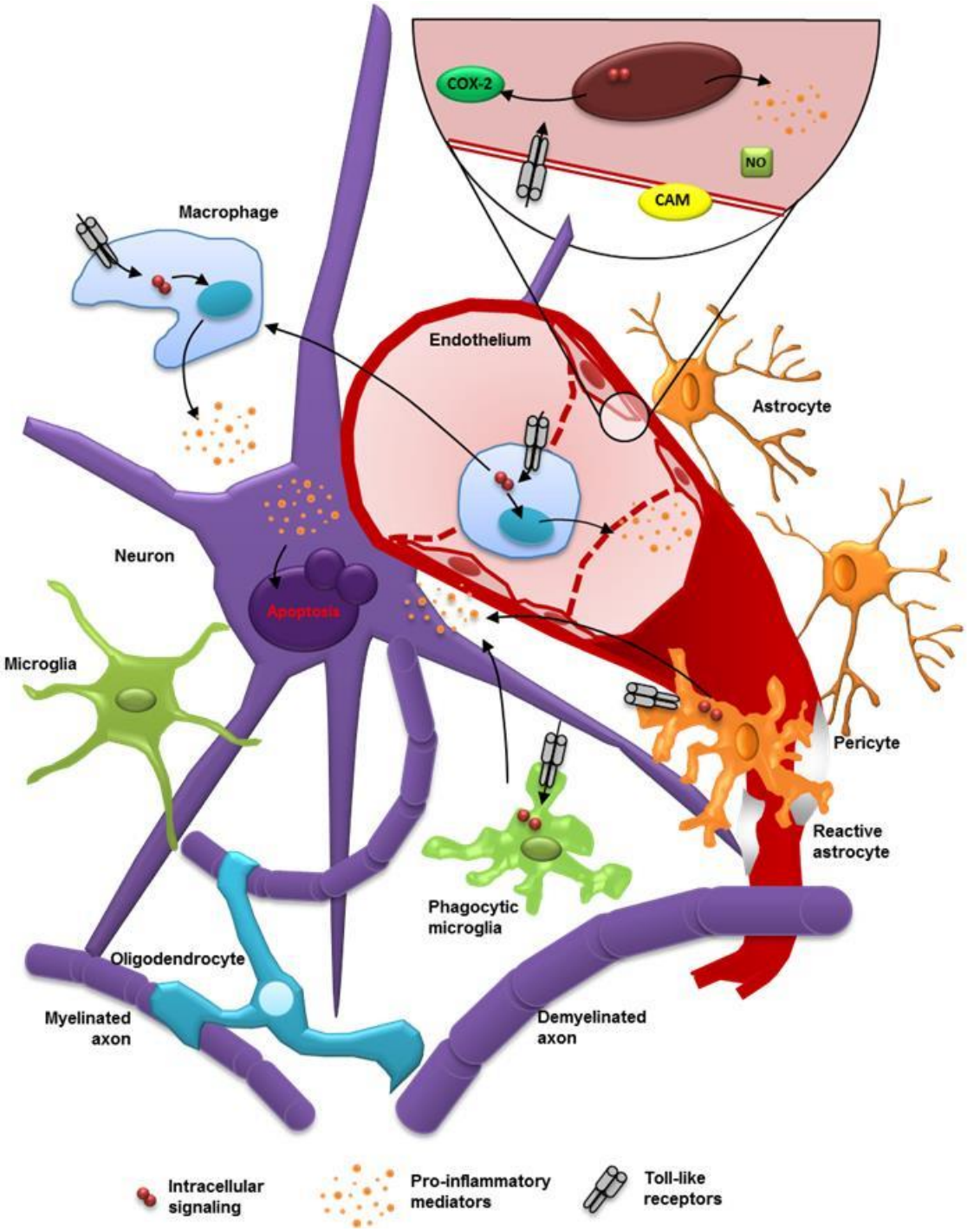


**Figure 1.15 - Main alterations occurring at the neurovascular unit during neuroninflammation associated to sepsis.** The activation of Toll-like receptors in endothelial cells and in circulating immune cells increases the production of cyclooxygenase (COX)-2, vasodilator nitric oxide (NO), and proinflammatory mediators, affecting the blood-brain barrier permeability and promoting the expression of cellular adhesion molecules (CAM). This would lead to the subsequent activation of other central nervous system resident cells, including microglia and astrocytes, which will further promote the production of pro-inflammatory mediators and contribute to neurodegeneration and demyelination. Adapted from (Drouin-Ouellet and Cicchetti 2012).

### *4.2.3. Sepsis as an aggravating factor of jaundice*

Sepsis is an important risk factor for neonatal hyperbilirubinemia or neonatal neurotoxicity (Gamaleldin et al. 2011; van Imhoff et al. 2011). Though clinical evidence is limited, it has been demonstrated that the neurotoxicity of UCB is amplified by the simultaneous exposure to LPS. In astrocytes simultaneously exposed to UCB and LPS, there is an over secretion of pro-inflammatory cytokines as well as an aggravation of necrosis-like and apoptotic cell death versus exposure to UCB alone, an effect that is sustained in more immature cells (Falcão et al. 2005; Fernandes et al. 2004). Neurons are also particularly susceptible to UCB and LPS damage. Pre-exposure to UCB followed by an inflammatory stimulus leads to an exacerbated apoptosis and to a greater neuritic breakdown (Falcão et al. 2007). Hence, LPS potentiates the immunostimulatory properties of UCB which, alongside with the exacerbation in glial immature cells, might thus point to the increased susceptibility of premature newborns to hyperbilirubinemia effects when associated with sepsis. Up to date, little is known about the effects induced on BMEC by joint exposure to these two neurotoxins.

Altogether, developing prenatal/neonate inflammatory response can subsequently influence brain development. Nonetheless, few studies focus on the acute and lasting behavioral and cognitive consequences of an inflammatory insult during the embryonic or perinatal period.

## 5. Global aims of the thesis

The main goal of the present work was to explore the effects of two frequent neonatal conditions, hyperbilirubinemia and systemic inflammation, on the developing neurovascular unit through the administration of UCB and/or LPS.

As a first step we aimed to investigate the acute and delayed cellular effects of UCB and LPS, alone or in combination, on the brain microvascular endothelium. Therefore we intended to evaluate the functioning of the endothelium by assessing cell viability as well as transport and efflux pathways in BMEC. We additionally sought to explore the effects on the integrity of BMEC by evaluating the structure of organelles and intercellular junctions, the latter also in co-cultures with astrocytes.

Given that astrocytes worsened the integrity of BMEC in co-cultures exposed to LPS, we further aimed to explore the acute effects of this endotoxin in the premature neurovascular unit. Thus we intended to evaluate cerebellar morphology, possible neuronal and white matter defects, as well as the response of inflammatory biomarkers. For deeper understanding of this neonatal condition we planned to study the processes of myelination, reactivity of glial cells and their interaction with the endothelium in CX3CR1$^{gfp/+}$ transgenic mice.

Lastly, the presence of acute-to-delayed damaging effects induced by LPS on the premature neurovascular unit, led us to further dissect the acute impact of neuroinflammation in the term brain. In addition, we intended to determine in young adult mice if the consequences were lasting and whether they were dependent of the neonatal age of LPS-treatment. We also aimed to determine the results of neonatal priming in the response to a second inflammatory stimulus.

All together, we expect that the present study will contribute to elucidate the causes behind the sequelae observed in sepsis survivors, in order to define possible targets and timing of novel therapeutic strategies. Indeed, we believe that neuroprotective therapies will only be fully successful by understanding the different mechanisms involved in early and late neonatal inflammatory responses.

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*Chapter 2*

# EXPOSURE TO LIPOPOLYSACCHARIDE AND/OR UNCONJUGATED BILIRUBIN IMPAIR THE INTEGRITY AND FUNCTION OF BRAIN MICROVASCULAR ENDOTHELIAL CELL

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## Abstract

Sepsis and jaundice are common conditions in newborns that can lead to brain damage. Though lipopolysaccharide (LPS) is known to alter the integrity of the blood-brain barrier (BBB), little is known on the effects of unconjugated bilirubin (UCB) and even less on the joint effects of UCB and LPS on brain microvascular endothelial cells (BMEC). Monolayers of primary rat BMEC were treated with 1 mg/ml LPS and/or 50 μM UCB, in the presence of 100 μM human serum albumin, for 4 or 24 h. Co-cultures of BMEC with astroglial cells, a more complex BBB model, were used in selected experiments. LPS led to apoptosis and UCB induced both apoptotic and necrotic-like cell death. LPS and UCB led to inhibition of P-glycoprotein and activation of matrix metalloproteinases-2 and -9 in monocultures. Transmission electron microscopy evidenced apoptotic bodies, as well as damaged mitochondria and rough endoplasmic reticulum in BMEC by either insult. Shorter cell contacts and increased caveolae-like invaginations were noticeable in LPS-treated cells and loss of intercellular junctions was observed upon treatment with UCB. Both compounds triggered impairment of endothelial permeability and transendothelial electrical resistance both in mono- and co-cultures. The functional changes were confirmed by alterations in immunostaining for junctional proteins b-catenin, ZO-1 and claudin-5. Enlargement of intercellular spaces, and redistribution of junctional proteins were found in BMEC after exposure to LPS and UCB. LPS and/or UCB exert direct toxic effects on BMEC, with distinct temporal profiles and mechanisms of action. Therefore, the impairment of brain endothelial integrity upon exposure to these neurotoxins may favor their access to the brain, thus increasing the risk of injury and requiring adequate clinical management of sepsis and jaundice in the neonatal period.


*Keywords:* blood-brain barrier; endothelial cells; lipopolysaccharide; unconjugated bilirubin; astrocytes; P-glycoprotein; metalloproteinases; tight junctions; adherens junctions; transmission electron microscopy.

## 1. Introduction

The blood-brain barrier (BBB) is a dynamic interface between blood and brain compartments that protects nerve tissue from insults. Brain microvascular endothelial cells (BMEC), possessing unique properties, are considered the main constituents of the barrier. They regulate the selective passage of substances through the expression of specific influx and efflux transport systems (Cardoso et al. 2010). ATP-binding cassette (ABC) transporters, such as the efflux transporter P-glycoprotein (P-gp), export potentially toxic compounds. A relevant transcellular vesicular transport mechanism at the BBB occurs through caveolae, which are dynamic pieces of membrane enriched in cholesterol and sphingolipids, as well as in the structural protein caveolin-1 (Cardoso et al. 2010). Additionally, BMEC display cohesive intercellular junctional complexes, composed of tight junctions (TJs) and adherens junctions (AJs). TJs are formed by transmembrane proteins like claudins, occludin, tricellulin, junctional adhesion molecules, and cytoplasmic proteins, like the zonula occludens (ZO) family (Mariano et al. 2011). TJs are responsible for high transendothelial electrical resistance (TEER) and low paracellular permeability at the BBB (Deli 2009, Wolburg and Lippoldt 2009). AJs are constituted by the transmembrane proteins vascular endothelial cadherins, nectins, platelet-endothelial cell adhesion molecule, and by the cytoplasmic catenins, comprising β-catenin (Niessen 2007). BMEC, pericytes and astrocytes share a thick basement membrane that is composed of various extracellular matrix (ECM) classes of molecules (Cardoso et al. 2010). Matrix metalloproteinases (MMPs) are known to digest basement membrane proteins and impair TJs integrity (Cardoso et al. 2010). Pathological conditions affecting the integrity of intercellular junctions, BBB transporters or the basement membrane impair the barrier function of the BBB, which can lead to or further increase brain damage.

Sepsis reflects an uncontrolled systemic inflammatory response to an infection that can cause organ dysfunction, eventually leading to shock or even death (Akrout et al. 2009). Lipopolysaccharide (LPS) is the major component of the outer membrane of Gram-negative bacteria. It may circulate in low levels in the blood in certain diseases (Dohgu and Banks 2008), but high levels suggest infection or sepsis. Rat BMEC have been shown to express the Toll-like receptors (TLR) 2, 3, 4, 6 and the membrane cluster of differentiation 14 (CD14), which binds LPS (Nagyõszi et al. 2010, Singh and Jiang 2004). When activated, these receptors trigger the release of pro-inflammatory cytokines into the brain parenchyma and induce neuroinflammation. Our previous studies have shown that binding of LPS to rat primary BMEC co-cultured with astrocytes leads to increased permeability, reduced TEER, alterations in intercellular

junctions assembly, as well as to inhibition of P-gp activity (Veszelka et al. 2007). These changes in BBB integrity may favor the access of neurotoxins as well as of microbial pathogens to the brain (Dohgu and Banks 2008).

Unconjugated bilirubin (UCB), the principal end product of heme catabolism, circulates in the plasma almost entirely bound to albumin due to its low solubility in aqueous medium, and the concentrations of unbound (free) bilirubin are in the nM range (Brites and Brito 2012). At low or slightly elevated concentrations as those reported for Gilbert patients, who present up to 100 μM total serum bilirubin and a UCB to albumin molar ratio of 0.2, UCB is a powerful antioxidant, able to provide protection against cardiovascular diseases and cancer (Brito et al. 2006, Vítek et al. 2002, Yesilova et al. 2008). It was also shown that UCB formed by upregulation of heme oxygenase-2, which is constitutive and highly concentrated in neurons, protects these nerve cells from $H_2O_2$-induced loss of cell viability. However, at higher concentrations, UCB is no longer beneficial but instead induces neuronal death (Doré et al. 1999). This dual behavior was observed in our own laboratory, where 10 nM free UCB were shown to protect neurons from $H_2O_2$-induced neuronal death, nuclear factor (NF)-κB activation and tumor necrosis factor (TNF)-α secretion, whereas 100 nM was neurotoxic (Gualazzi et al. 2007). These observations support the notion that UCB acts as a double-edged sword, either beneficial at low concentrations, or detrimental at elevated levels. Accordingly, in the experimental conditions that we have been consistently using with different cell types (Falcão et al. 2005, Fernandes et al. 2009, Gordo et al. 2006), which reproduce a moderate or severe hyperbilirubinemia [UCB to human serum albumin (HSA) molar ratios of 0.5 and 1.0, respectively], toxicity is observed and oxidative stress appears as a relevant event (Brito et al. 2004, Brito et al. 2008a, Vaz et al. 2010). Hyperbilirubinemia is particularly common in the neonatal period due to the shorter life span of fetal erythrocytes, together with the immaturity of the hepatic clearance system (Brito et al. 2006). These high levels of UCB become neurotoxic by inducing oxidative and nitrosative stress, mainly in neurons (Brito et al. 2008b, Brito et al. 2010), and the release of pro-inflammatory cytokines by astrocytes (Fernandes et al. 2007) and microglia (Silva et al. 2010). Our most recent studies revealed that human BMEC exposed to UCB are also under nitrosative stress, as indicated by the upregulation of endothelial nitric oxide synthase and production of nitrites, the end-product of nitric oxide, and release cytokines, such as the vascular endothelial growth factor and interleukin (IL)-6 (Palmela et al 2011). Interestingly, both nitric oxide and these cytokines are known to increase BBB permeability (de Vries et al. 1996, Deli et al. 2005). Despite such indication of a hyperpermeability of the BBB upon exposure to UCB, direct evidence of the disruption of the barrier properties by UCB exposure has

never been provided. Hyperbilirubinemia and sepsis frequently occur as associated pathological conditions in newborn infants. Sepsis is considered as a neurotoxicity risk factor during neonatal hyperbilirubinemia (Maisels et al. 2009) and a recent prospective study indicated that jaundiced infants with encephalopathy were more likely to have a coexistent infection than hyperbilirubinemic infants without brain damage (Manning et al. 2007). Our previous studies showed that the inflammatory response and cell death in astrocytes are exacerbated by LPS (Falcão et al. 2005, Fernandes et al. 2004). However, the effects produced by the combination of these compounds have never been studied in BMEC. The aim of this study was to reveal the effects of LPS and UCB, alone or in combination, on the morphological and functional characteristics of rat BMEC in conditions mimicking sepsis and moderate to severe hyperbilirubinemia.

## 2. Materials and Methods

### 2.1. Cell Cultures

Animal care followed the recommendations of European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes (Council Directive 86/ 609/EEC) and National Law 1005/92 (rules for protection of experimental animals). Formal approvals to conduct all animal procedures in the experiments have been obtained from the Animal Experimentation Committee of the Biological Research Centre, Hungarian Academy of Sciences (Hungary), and from the local authorities (Permit number: XVI./03835/001/2006).

Primary cultures of rat BMEC were prepared from 2-week-old Wistar rats, as previously described (Kis et al. 2001, Perrière et al. 2005, Veszelka et al. 2007). Briefly, meninges were removed from forebrains and gray matter was minced into small pieces in Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 (DMEM/F12, Biochrom AG, Germany), and digested in DMEM/F12 containing 1 mg/ml collagenase CLS2 (Worthington, USA) for 1.5 h at 37ºC. Cell pellet was separated by centrifugation in 20% bovine serum albumin-DMEM/F12 (1000x*g*, 20 min). Microvessels were further digested with 1 mg/ml collagenase-dispase (Roche Applied Sciences, Switzerland) in DMEM/F12 for 1 h at 37ºC. Microvessel endothelial cell clusters were separated on a 33% continuous Percoll (Pharmacia, Sweden) gradient, collected and washed in DMEM/F12 before plating ($1.5x10^5$ cells/$cm^2$) onto collagen type IV and fibronectin coated cell culture inserts or multiwell plates. Cultures were maintained in DMEM/F12 supplemented with 15% fetal bovine serum (FBS) (Biochrom AG), 1 ng/ml basic fibroblast growth factor (Roche Applied Sciences), 100 μg/ml heparin (Biochrom AG), 5 μg/ml gentamycin and 4 μg/ml puromycin (Perrière et al. 2005) (medium I) at 37ºC

with a humidified atmosphere of 5% $CO_2$, for 2 days. On day 3, cells received new medium, which contained all components of medium I except puromycin (medium II).

Primary cultures of glial cells were prepared from newborn Wistar rats (Kis et al. 2001, Perrière et al. 2005, Veszelka et al. 2007). Meninges were removed, and cortical pieces were mechanically dissociated in DMEM containing 5 mg/ml gentamicin and 10% fetal bovine serum and plated in poly-L-lysin coated 12-well dishes and kept for minimum 3 weeks before use. In confluent astroglia cultures 90% of cells were immunopositive for the astroglia cell marker glial fibrillary acidic protein, while the remaining 10% was immunopositive for CD11b, a marker of microglia.

For co-culture, BMEC in cell culture inserts were placed into multiwells containing glia at the bottom of the wells with endothelial culture medium in both compartments. When BMEC became almost confluent 550 nM hydrocortisone was added to the culture medium (Hoheisel et al. 1998), for 1 day.

### 2.2. Treatment of Endothelial Cells

UCB (Sigma-Aldrich, USA), was purified according to the method of McDonagh and Assisi (1972) and stock solutions were extemporarily prepared in 0.1 M NaOH, under light protection, and the pH adjusted to 7.4 by addition of equal amounts of 0.1 M HCl. A stock solution of LPS (Escherichia coli O111:B4; Calbiochem, Germany) was prepared in PBS. Confluent monolayers of rat BMEC received medium II with 2% FBS, and were divided into four groups: untreated cells (control), 1 μg/ml LPS, 50 μM UCB, in the presence of 100 μM human serum albumin (fraction V, fatty acid free, Sigma-Aldrich), or a combination of both, for 4 h and 24 h at 37ºC. The concentration of LPS was selected to mimic sepsis (Cepsinskas et al. 2008, Tien et al. 2010, Wang et al. 2004) and was shown to induce damage to rat BMEC in our previous study (Veszelka et al. 2007). The use of albumin mimics the *in vivo* condition, where there is equilibrium between the bilirubin fraction that is bound to albumin, the one that is free in circulation and the one that binds to cells. Therefore, albumin functions as a sink for bilirubin, so that bilirubin may be continuously released from albumin as it binds to cells, therefore perpetuating the fraction that is available for interaction with cells and thus enhancing the effects. The UCB condition mimics moderate to severe hyperbilirubinemia in a term or a preterm infant, respectively (Ahlfors et al. 2003). In fact, studies performed in our lab showed that a group of full term neonates with moderate jaundice present a molar ratio of 0.42 (~195 μM bilirubin and ~445 μM serum albumin) (Brito, 2001), and that a preterm infant with a bilirubin to albumin molar ratio of 1.0 (~493 μM bilirubin and ~498 μM serum albumin) at the second day of life dyed on the fourth with the diagnosis of kernicterus (Brito et al. 2012). In the present experimental conditions, the concentration

of unbound UCB (free UCB), determined by the peroxidase method (Roca et al. 2006), was 12 nM, which is close to the 19 nM value found in the moderate jaundiced infants studied (Brito 2001). The absence of biliverdin in the UCB-treated samples was assured by continuous absorbance spectra from 300 to 800 nm (Maghzal et al. 2009), which revealed no peaks at 380 and 665 nm.

### 2.3. Cell Death Evaluation

Standard evaluation of rat BMEC cytotoxicity was performed by measuring the release of LDH from cells with damaged plasma membrane into the incubation medium using a cytotoxicity detection kit (Roche Molecular Biochemicals, Germany), as previously described (Cepsinskas et al. 2008). The reaction was performed in a 96-well microplate and the absorbance measured at 490 nm, using a PR 2100 microplate reader from Bio-Rad (USA). All readings were corrected for possible interference of UCB absorption and the results expressed as percent of LDH release. Cytotoxicity was calculated as percentage of the total LDH release from untreated cells lysed with 2% Triton X-100 for 30 min. To evaluate apoptosis, cells fixed in freshly prepared 4% paraformaldehyde solution in phosphate buffer saline (PBS) were immunostained with Hoechst 33258 dye for 2 min at room temperature, and mounted in Glycerol Mount. Apoptotic nuclei were identified by condensed chromatin or nuclear fragmentation, and counted for each independent experiment in at least five random microscopic fields (400x) per sample, as described previously (Fernandes et al. 2004), using a Leica DFC 490 camera (Leica, Germany) adapted to an AxioScope.A1 microscope (Zeiss, Germany). Although the fluorescence emission
of Hoescht 33258 dye at 461 nm overlaps with the absorption of UCB at 460 nm, the possible quenching by UCB absorption does not interfere with the obtained results since apoptosis was evaluated based on morphology and not on fluorescence intensity. Activity of caspase-3 was measured by a colorimetric method (Calbiochem, Darmstadt, Germany), as usual in our lab (Palmela et al. 2011). Briefly, cells were harvested, washed with ice-cold PBS, and lysed for 30 min, on ice, in the lysis buffer [50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4); 100 mMNaCl; 0.1% (w/v) cholamidopropyldimethylammonio-1-propanesulfonate; 1 mM dithiothreitol; 0.1 mM ethylenediaminetetraacetic acid]. The lysate was centrifuged at 10 000x*g* for 10 min at 4ºC and the supernatants were collected and stored at -80ºC. The activity of caspase-3 was determined by enzymatic cleavage of chromophore pNA from the substrate, according to manufacturer's instructions. The proteolytic reaction was carried out in protease assay buffer [50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4); 100 mM NaCl; 0.1% (w/v) cholamidopropyldimethylammonio-1-propanesulfonate;

10 mM dithiothreitol; 0.1 mM ethylenediaminetetraacetic acid; 10% (v/v) glycerol], containing 2 mM substrate Ac-DEVD-pNA. Following incubation of the reaction mixtures for 2 h at 37ºC, the formation of pNA was measured at 405 nm with a reference filter of 620 nm. The results were expressed as relative activity *vs*. control samples.

### 2.4. Functional Assay for P-gp

Activity of P-gp was determined by measuring cellular accumulation of the P-gp substrate R123 per mg of protein content (Veszelka et al., 2007), and results were expressed as fold-change as compared to the respective control. In brief, treated rat BMEC were washed, and incubated for 1 h at 37ºC with Ringer–Hepes solution (118 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl2, 1.2 mM MgSO4, 5.5 mM D-glucose, 20 mM Hepes, pH 7.4) containing 10 μM R123. The solution was quickly removed, rat BMEC were washed three times with PBS and solubilized in 0.1 M NaOH. R123 content was determined using a Polarstar Galaxy fluorescent plate reader (BMG Labtechnologies; excitation at 505 nm, emission at 538 nm). The fluorescence emission of R123 is not quenched by bilirubin's absorption, as the absorption of UCB is at 460 nm. Verapamil (100 μM, 30 min pre-incubation) was used as a reference P-gp inhibitor for the positive control (1.9-fold at 4 h and 2.8-fold at 24 h). Protein content was evaluated by the Bradford method (Bradford 1976) using Bio-Rad's Protein Assay reagent (Bio-Rad).

### 2.5. Gelatin Zymography

Determination of MMP-2 and MMP-9 was evaluated as previously described (Silva et al. 2010). In short, aliquots of culture supernatants were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) zymography in 0.1% gelatine–10% acrylamide gels under non-reducing conditions. After electrophoresis, gels were washed for 1 h with 2.5% Triton X-100 (in 50 mMTris pH 7.4, 5 mM $CaCl_2$, 1 μM $ZnCl_2$) to remove SDS and renature the MMPs species in the gel. Then the gels were incubated in the developing buffer (50 mM Tris pH 7.4, 5 mM $CaCl_2$, 1 μM $ZnCl_2$) overnight to induce gelatin lysis. For enzyme activity analysis, the gels were stained with 0.5% Coomassie Brilliant Blue R-250 (Bio-Rad) and destained in 30% ethanol/10% acetic acid/$H_2O$. Gelatinase activity, detected as a white band on a blue background, was quantified by computerized image analysis and normalized with total cellular protein using Quantity One 1-D Analysis Software (Bio-Rad).

### 2.6. TEM Analysis

Rat BMEC mono-cultures grown on inserts were fixed with freshly prepared 4% paraformaldehyde in 0.05 M cacodylate buffer (pH 7.5) for 30 min at 4ºC. After washing with cacodylate buffer several times, the membranes of the cell culture inserts with the cells were removed from their support and placed into 24-well chamber slide and were post-fixed in 1% $OsO_4$ for 30 min. Following washing with distilled water, the cells on the membrane were dehydrated in graded ethanol, block-stained with 1% uranyl acetate in 50% ethanol for 30 min and embedded in Taab 812 (Taab; Aldermaston, UK). Following polymerization at 60ºC for 12 h, 50–60 nm ultrathin sections were cut perpendicularly for the membrane using a Leica UCT ultramicrotome (Leica Microsystems, UK) and examined using a Hitachi 7100 transmission electron microscope (Hitachi Ltd., Japan). Electron micrographs were made by Veleta, a 2κ x 2κ MegaPixel side-mounted TEM CCD camera (Olympus). Electron micrographs were edited by Adobe Photoshop CS3 (Adobe Photoshop Incorporation, CA, USA).

### 2.7. Evaluation of the Monolayer Integrity

The flux of Na-F (MW: 376 Da) across endothelial monolayers and co-cultures was determined as previously described (Kis et al. 2001, Veszelka et al. 2007). Briefly, cell culture inserts were transferred to 12-well plates containing 1.5 ml Ringer–Hepes solution in the abluminal compartments. Culture medium from luminal compartments was replaced by 0.5 ml Ringer–Hepes solution containing 10 μg/ml Na-F. Inserts were transferred at 20, 40 and 60 min to new wells containing Ringer–Hepes solution. Na-F levels were measured using a Hitachi F-2000 fluorescence spectrophotometer (excitation: 440 nm, emission: 525 nm). Flux across cell-free inserts was also measured. Endothelial permeability coefficient was calculated as previously described (Deli et al. 2005, Veszelka et al. 2007) and results were expressed in cm/s or as fold-change compared to the respective control. TEER of mono-cultures and co-cultures, reflecting the paracellular permeability for mainly sodium ions in the present culture conditions, was measured using a STX-2 electrode coupled to an EVOM resistance meter (World Precision Instruments, USA). TEER readings of cell-free inserts (~100 Ω $cm^2$) were subtracted from the values obtained with cells. For comparison of the data between mono-cultures and co-cultures TEER of cells treated with no addition (control) was considered 100%.

### 2.8. Immunostaining for Intercellular Junctions

BMEC in mono-culture or co-cultured with astrocytes were stained for junctional proteins ZO-1, claudin-5 and β-catenin. Cultures were washed in PBS and fixed with

ethanol (95%)–acetic acid (5%) (v/v) for 10 min at -20ºC (Veszelka et al. 2007). Cells were blocked with 3% BSA and incubated with primary antibodies anti-ZO-1 (1:200), anti-claudin-5 (1:200), and anti-β-catenin (1:200) (Invitrogen, USA) overnight at 4ºC. Incubation with secondary antibody Cy3-labelled anti-rabbit IgG (1:200) (GE Healthcare, UK) and Alexa 488 anti-mouse IgG (1:500) (Invitrogen) lasted for 1 h at room temperature. Nuclei were counterstained with Hoechst 33258 dye. Between incubations cells were washed three times with PBS. Coverslips were mounted in Gel Mount (Biomeda, USA) and staining was examined by a Nikon Eclipse TE2000 fluorescent microscope (Nikon, Japan) and photographed using a Spot RT digital camera (Diagnostic Instruments, USA) or using a Leica DFC 490 camera (Leica, Germany) adapted to an AxioScope.A1 microscope (Zeiss, Germany).

### 2.9. Statistical Analysis

Results of at least three different experiments were expressed as mean ± S.E.M. Differences between groups were determined by one-way ANOVA using Prism 5.0 (GraphPad Software, San Diego, CA) followed by multiple comparisons Bonferronipost-hoc correction. Statistical significance was considered when *P* values were lower than 0.05.

## 3. Results

### 3.1. LPS, UCB and their Combination Induce Cell Death in Rat BMEC

Primary cultures of rat BMEC are commonly used as simplified *in vitro* models of the BBB (for review see Deli et al. 2006 and Tóth et al. 2011). In order to evaluate the impairment of rat BMEC viability by exposure to LPS and UCB, we assessed necrosis-like cell death by determining the release of intracellular lactate dehydrogenase (LDH) into the incubation medium. As seen in Fig. 2.1A, LPS triggered a mild, though not statistically significant, elevation of LDH release, which attained maximum levels of 9.4% and 16.2% at 4 and 24 h, respectively, corresponding to an overall time-dependent elevation ($P<0.01$) in the extent of cell death. With UCB, higher values were achieved (16.9% at 4 h and 21.4% at 24 h, $P<0.01$ and $P<0.05$, respectively, as compared to the respective control), reflecting a time-dependent loss of cell viability ($P<0.05$). There was no aggravation in necrosis-like cell death when cells were exposed to both LPS and UCB (LDH release at 24 h: 20.3%, $P<0.05$ as compared to the respective control), even though it similarly increased with the time of exposure ($P<0.01$).

Either UCB or LPS induced apoptosis in BMEC, as observed by staining endothelial nuclei with Hoechst 33258 dye followed by analysis of nuclear morphological features (Figs. 2.1B,C), in accordance with previous demonstrations (Palmela et al. 2011, Shiori et al. 2009). LPS induced apoptotic cell death in a time-dependent manner ($P$<0.01), reaching 18.0% ($P$<0.05) after 24 h treatment. When exposed to UCB, the percentage of cells with apoptotic morphology was already significantly increased at 4 h (19.6%, $P$<0.001) and sustained at 24 h (20.7%, $P$<0.001). As for LDH, there was no increment in UCB-induced apoptotic cell death by co-incubation with LPS. In contrast, apoptosis raised from 13.1% in LPS-treated cells to 18.7% in cells treated with both LPS and UCB for 4 h ($P$<0.01 as compared to LPS alone). To note that this difference disappeared as the incubation was extended to 24 h and death continued to increase in cells treated with LPS alone.

We also studied the activity of caspase-3 (Fig. 2.1D). LPS induced no significant alterations in caspase-3 activity at the time-points studied, reaching a maximum elevation of 1.2-fold at 4 h. On the other hand, cells exposed to UCB had a significant increase in caspase-3 activity at the earliest time-point, attaining 1.9- and 2.0- folds for UCB and UCB with LPS, respectively ($P$<0.05). At 24 h cells treated with UCB alone revealed a sustained caspase-3 activity (1.7-fold, $P$<0.05).

### 3.2. Activity of ABC Transporter P-gp is Impaired by LPS and UCB

We studied the effects of LPS and UCB on the efflux transporter P-gp activity (Fig. 2.2), based on the measurement of cellular accumulation of rhodamine 123 (R123), the main substrate dye used to assess P-gp function. Accumulation of the substrate reflects a decreased activity of the efflux transporter, as have been recognized and used, particularly in primary cultures of BMEC (Fontaine et al. 1996, Veszelka et al. 2007). Results were expressed as fold-change from control, which presented ~114 ng R123/mg protein. P-gp activity was already diminished following 4 h exposure to LPS or UCB, as indicated by the 1.2-fold elevation in intracellular levels of R123 ($P$<0.05). Exposure to both LPS and UCB increased P-gp inhibition with time ($P$<0.05) and aggravated the inhibition induced by LPS alone at 24 h (1.2-fold for LPS *vs*. 1.4-fold for LPS plus UCB, $P$<0.05).

### 3.3. Activities of MMP-2 and MMP-9 are Increased by LPS and UCB

It has been known that the release of active MMPs is related to the opening of the BBB in LPS-injured brain tissue (Lee et al. 2011). The activities of MMP-2 and MMP-9 released by rat BMEC after exposure to the insults were similar (Fig. 2.3). Significant increases in the activity of secreted MMPs were already observed at 4 h for both LPS

(1.7-fold for MMP-9 and 1.8-fold for MMP-2, *P*<0.05) and UCB (1.9-fold for MMP-9 and MMP-2, *P*<0.05), which were less marked at 24 h treatment though still statistically significant. No significant aggravation of the effect was observed by simultaneous exposure to both neurotoxins, either at 4 or at 24 h.

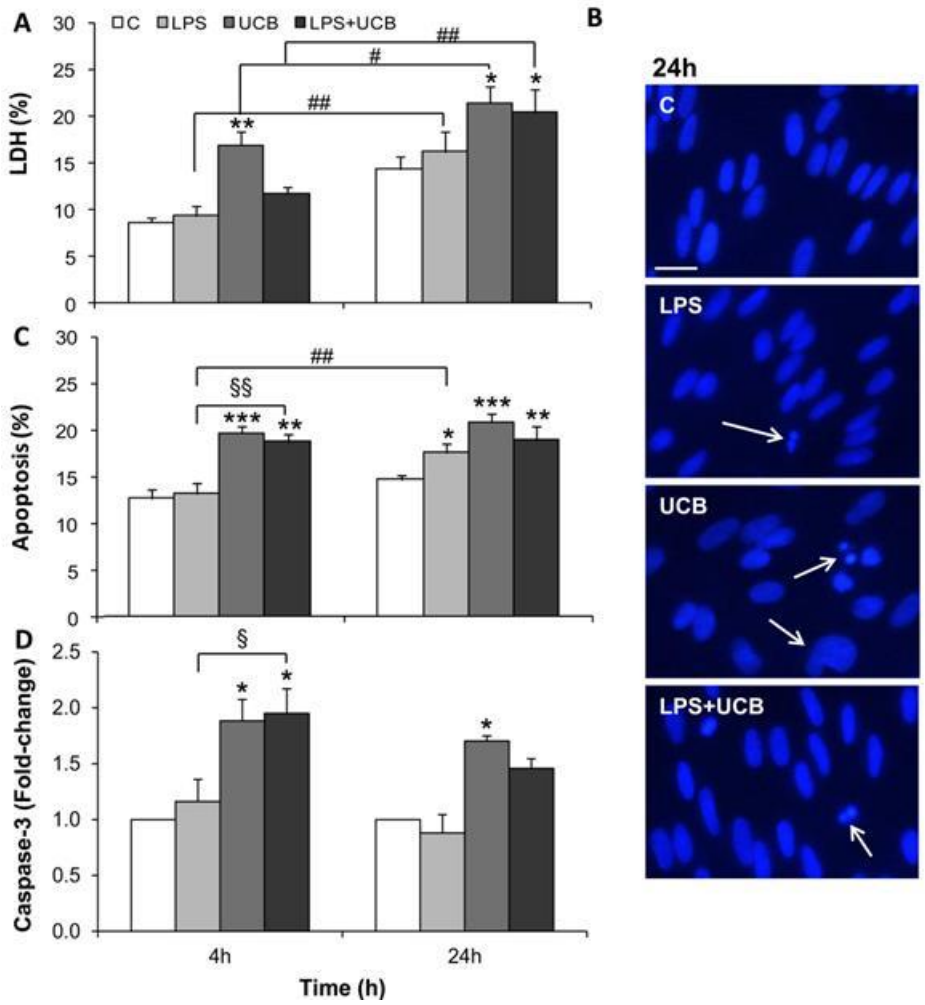


**Figure 2.1 – Lipopolysaccharide (LPS) and unconjugated bilirubin (UCB) induce cell death in brain microvascular endothelial cells.** Culture medium was collected for determination of lactate dehydrogenase (LDH) activity (A). Nuclei were stained with Hoechst 33258 dye and morphological features of apoptosis are pointed (arrows) (B). The number of apoptotic nuclei was counted and results were expressed as percentage of the total number of nuclei (C). Cell lysates were obtained for caspase-3 activity determination (D). Results are mean ± S.E.M. from at least five independent experiments performed in duplicate. Scale bar, 20 µm. *P<0.05, **P<0.01 and ***P<0.001 *vs* respective control; $^{\S}$P<0.05, $^{\S\S}$P<0.01 *vs* LPS at the same time-point; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 from 4 h.

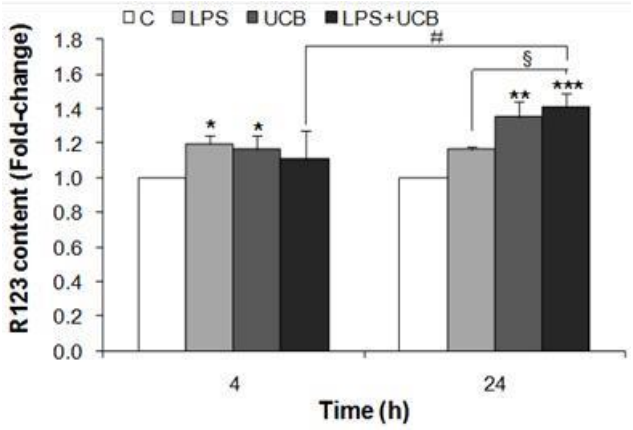


**Figure 2.2 – Lipopolysacharide (LPS) and unconjugated bilirubin (UCB) inhibit P-glycoprotein (P-gp) activity in brain microvascular endothelial cells.** To evaluate if P-gp is functioning properly, it was measured the protein's ability to remove the substrate rhodamine 123 (R123). After treatment, cells were incubated for 30 min with 10 μM R123. Total protein extracts were collected, R123 accumulation fluorescent emission was determined and results were expressed per mg of protein. Values presented are means ± S.E.M., from at least four independent experiments performed in duplicate. *P<0.05, **P<0.01 and ***P<0.001 *vs* respective control; $^{\S}$P<0.05 *vs* LPS at the same time-point; $^{\#}$P<0.05 from 4 h.

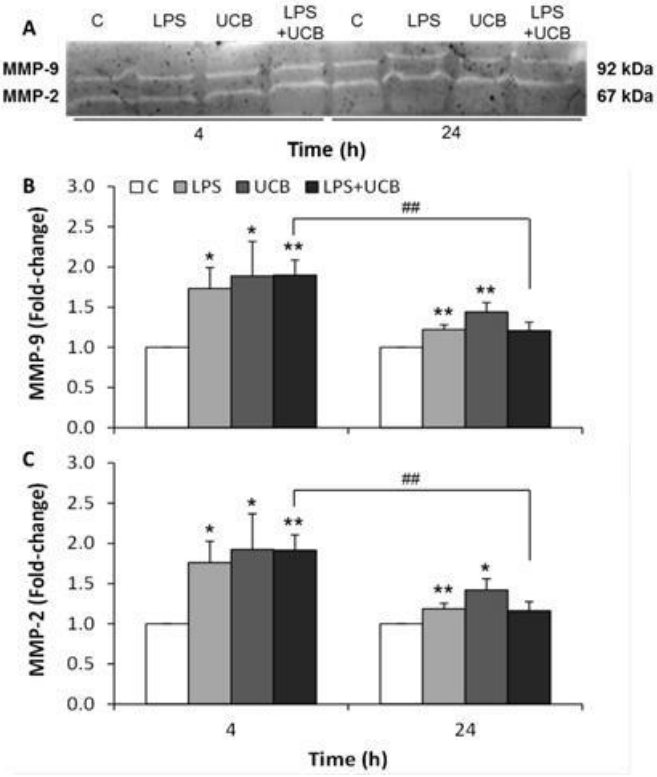


**Figure 2.3 – Lipopolysaccharide (LPS) and unconjugated bilirubin (UCB) activate metalloproteinase-9 (MMP-9) and metalloproteinase-2 (MMP-2) released by brain microvascular endothelial cells.** A representative gel from one experiment is shown, where MMP-2 and MMP-9 were identified by their apparent molecular mass of 67 and 92 kDa, respectively (A). The intensity of the bands was quantified by scanning densitometry and results for MMP-9 (B) and MMP-2 (C) were standardized with respect to total protein content. Results are mean ± S.E.M. from at least four independent experiments performed in duplicate. *P<0.05 and **P<0.01 *vs* respective control; $^{\#\#}$P<0.01 from 4 h.

### 3.4. LPS and UCB Alter Unique Ultrastructure of Rat BMEC

Transmission electron microscopy (TEM) investigated in detail the impairment of rat BMEC monolayers exposed to the neurotoxins (Fig. 2.4). In cell cultures treated with LPS or UCB, several signs of cell damage and the presence of apoptotic bodies released by rat BMEC were detected, confirming the occurrence of apoptotic processes. Our observations include heavily damaged mitochondria that were swollen and more electron dense than in control cells, indicating an impairment in mitochondrial function in both conditions. In addition, the rough endoplasmic reticulum was typically swollen in the treated cells. This alteration may indicate a lesion of the synthesis of lipids or metabolism of sugars, and also impairment in their detoxification function. Moreover, it was observed irregularly shaped vacuoles and holes in the cytoplasm, together with unevenness and little projections instead of a rather smooth, elongated shape of the rat capillary endothelial cell. Appearance and abundance of caveolae-like invaginations in the plasma membrane was frequently found in LPS-treated cells, an effect that was not so evident in cells incubated with UCB. TEM analysis revealed shortened extension of intercellular contacts in LPS-treated cells and no intact cell-cell junctions could be detected in UCB-treated cells.

### 3.5. Exposure to LPS and UCB Impair the Barrier Function of Rat BMEC

TEER and paracellular permeability to small molecule tracers provide information on barrier integrity in various *in vitro* BBB models (Deli et al. 2005, Tóth et al. 2011, Calabria and Shusta 2008). We have demonstrated that LPS treatment caused a concentration- and time-dependent decrease in TEER and increased permeability to tracers in rat BMEC co-cultured with astrocytes (Veszelka et al. 2007), but, so far, such features have not been explored in cells exposed to UCB or to both neurotoxins, either in co-cultures or in BMEC mono-cultures.

Permeability to sodium fluorescein (Na-F) was of ~$1.9 \times 10^{-5}$ cm/s in control BMEC monolayers. LPS exposure induced an elevation of permeability to $3.4 \times 10^{-5}$ cm/s at 4 h ($P<0.01$), and only a moderate increase after 24 h exposure ($2.1 \times 10^{-5}$ cm/s, $P<0.05$ *vs*. 4 h) (Fig. 2.5A). Similarly, cells treated with UCB showed an increased permeability at 4 h treatment ($4.0 \times 10^{-5}$ cm/s, $P<0.01$), with a sustained effect at 24 h. Alterations in permeability were significantly higher after simultaneous exposure to both insults at 24 h compared to those treated with LPS alone ($P<0.05$).

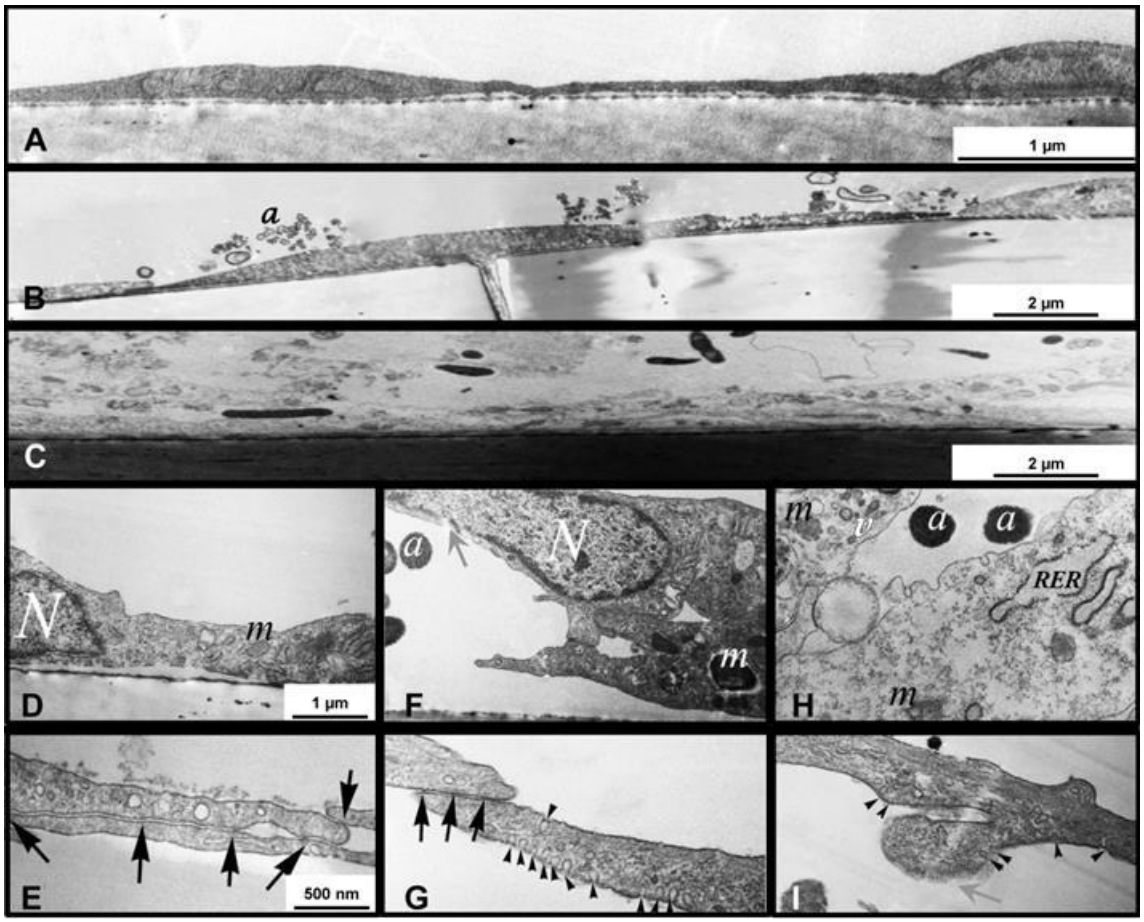


**Figure 2.4 – Lipopolysaccharide (LPS) and unconjugated bilirubin (UCB) disrupt ultrastructure of brain endothelial cells.** Cells were treated with no addition (control) (A,D,E), LPS (B,F,G) or UCB (C,H,I) and were analyzed by transmission electron microscopy. Black arrows, tight intercellular junctions; grey arrows, disruption of the plasma membrane; arrowheads, invaginations of the plasma membrane; a, apoptotic cells bodies; m, mitochondria; N, cell nuclei; RER, rough endoplasmic reticulum; v, vacuole. Representative results from one of four independent experiments are shown.

TEER was assessed after exposure to LPS and/or UCB for 4 h and 24 h (Fig. 2.5B), and results were compared with those obtained in controls (~42 $\Omega.cm^2$ after subtracting the values of cell free inserts). LPS treatment led to a significant decrease in TEER after 4 h exposure (20 $\Omega.cm^2$, $P<0.01$) and monolayers partially recovered from the early onset damage at 24 h, as indicated by higher resistance levels (36 $\Omega.cm^2$, $P<0.05$ *vs*. 4 h). On the other hand, UCB caused lower values through longer periods of exposure, as indicated by the TEER decrease at 4 h (24 $\Omega.cm^2$, $P<0.05$) and at 24 h (21 $\Omega.cm^2$, $P<0.001$). Incubation with both LPS and UCB for 24 h did not aggravate UCB-induced effect, but TEER values were significantly lower than for 24 h treatment with LPS alone ($P<0.01$ as compared to the respective control, and $P<0.05$ from LPS alone).

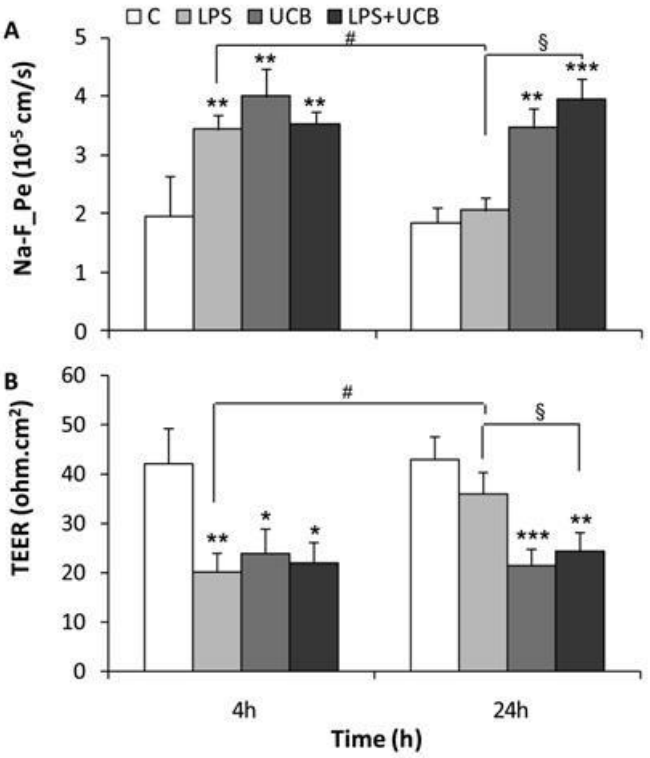


**Figure 2.5 – Lipopolysaccharide (LPS) and unconjugated bilirubin (UCB) disrupt the endothelial monolayer.** Permeability to sodium fluorescein (Na-F_Pe) (A) and transendothelial electrical resistance (TEER) (B) were determined after exposure. All values presented are means ± S.E.M. from at least four independent experiments performed in duplicate. *P<0.05, **P<0.01 and ***P<0.001 *vs* respective control; $^{\S}$P<0.05 *vs* LPS at the same time-point; $^{\#}$P<0.05 from 4 h.

Co-cultures of BMEC with astrocytes are accepted as more complex and tighter *in vitro* models of the BBB since astrocytes induce the barrier properties of BMEC (Cardoso et al. 2010, Deli et al. 2005, Tóth et al. 2011). To reinforce our observations, a set of experiments was performed in rat BMEC co-cultured with glial cells where the paracellular permeability to Na-F and the TEER were assessed 24 h after treatment (Fig. 2.6). In co-cultures, untreated BMEC had higher TEER (233 Ω.cm$^2$) and lower permeability (2.1x10$^{-6}$ cm/s) values than mono-cultures. The permeability pattern was similar to that of mono-cultures, though in general 2-fold greater and there was a significant increase induced by LPS. Regarding TEER, the response to the neurotoxins was also similar in both models, but the alterations resulting from the presence of astrocytes were more modest as compared with mono-cultures, as well as in comparison with those observed for permeability, particularly in UCB-treated cells.

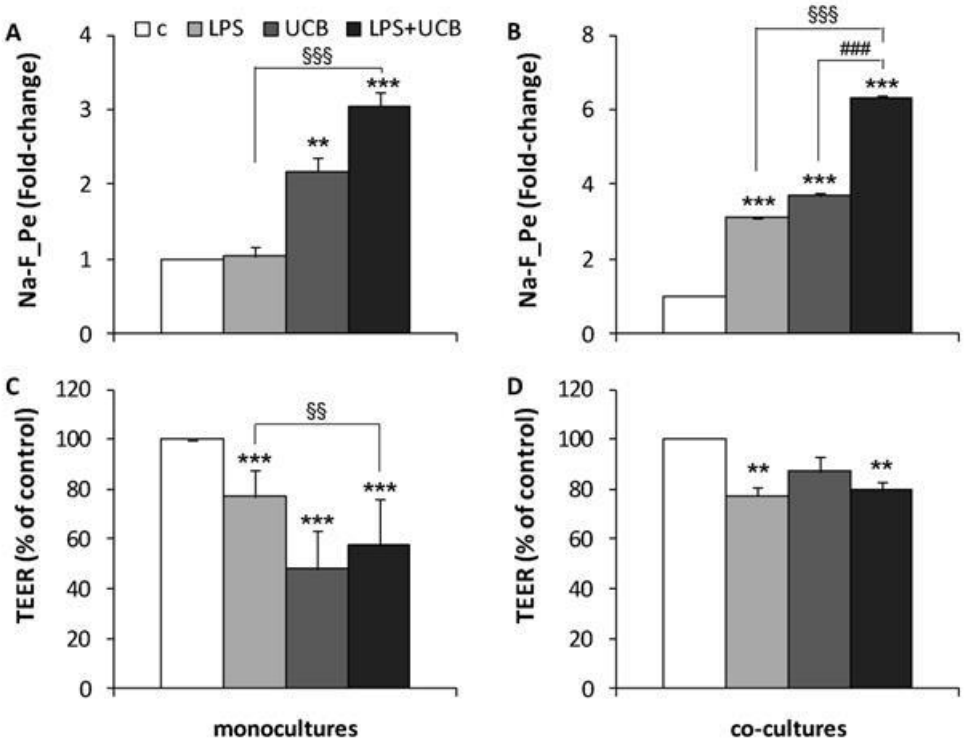


**Figure 2.6 – Effects of lipopolysaccharide (LPS) and unconjugated bilirubin (UCB) on endothelial integrity in mono-cultures and cocultures.** Permeability to sodium fluorescein (Na-F_Pe) (A, B) and transendothelial electrical resistance (TEER) (C, D) were determined in monocultures (A,C) and co-cultures (B,D) after 24 h exposure. All values presented are means ± S.E.M. from at least two independent experiments performed in triplicate. **P<0.01 and ***P<0.001 *vs* respective control; $^{§§}$P<0.01 and $^{§§§}$P<0.001 *vs* LPS at the same time-point.

### 3.6. Junctional Proteins in Rat BMEC are Altered by LPS and UCB

Cellular expression and localization of the AJ protein β-catenin was also assessed in mono-cultures both at 4 h and at 24 h (Fig. 2.7). After LPS treatment cell contours were hardly visible at 4 h, whilst β-catenin was relocated at the membrane at 24 h. β-catenin appeared as small dots in LPS-treated cells, which were particularly evident at 4 h incubation, contrasting with the normal staining observed in control cells. Treatment with UCB also revealed alterations in the distribution of β-catenin, which varied with the time of exposure. In fact, a short incubation led to a transient translocation of the protein from the membrane to the cytosol; this effect appears to be transient as at 24 h the presence of the protein was clearly noticed at intercellular contacts. Interestingly, an overall increase in fluorescence intensity of β-catenin was detected in cells treated for 24 h with UCB (1.3-fold from control, $P$<0.01), whereas a decrease was observed for LPS (0.7-fold, $P$<0.01). As a result of these opposite variations, no significant change in the fluorescence intensity was observed in cells simultaneously treated with LPS and UCB.

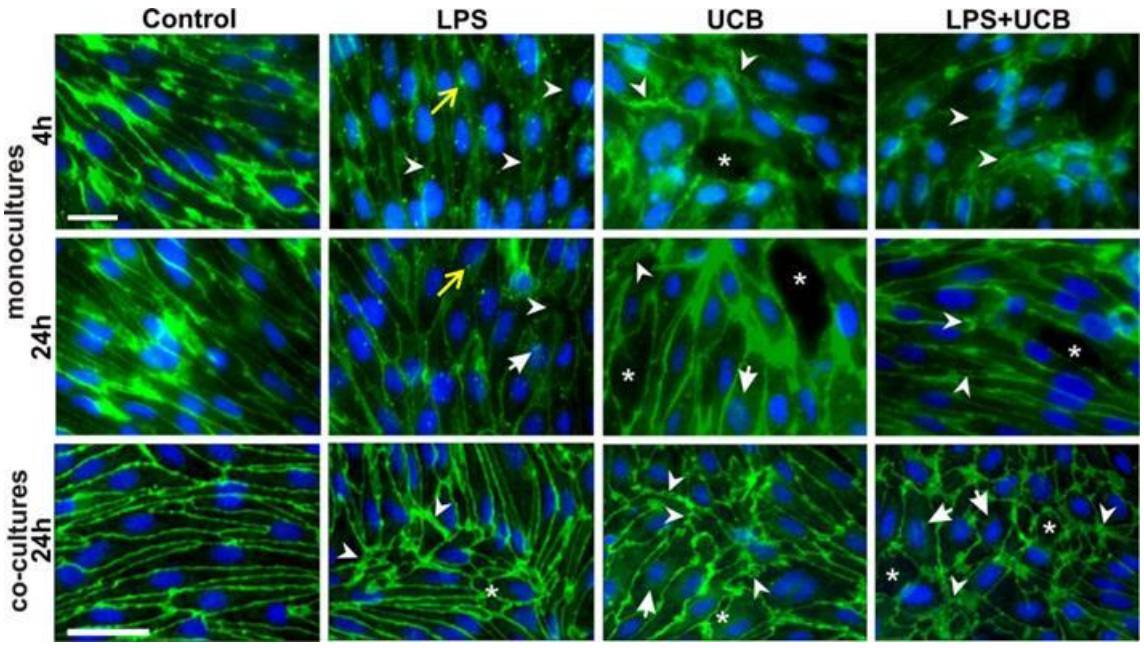


**Figure 2.7 – Lipopolysaccharide (LPS) and unconjugated bilirubin (UCB) modify the distribution of β-catenin in brain endothelial cells.** Cells in mono-culture or co-cultured with astrocytes were fixed and immunostained with an antibody against β-catenin to evaluate its cellular localization (scale bars, 40 and 20 µm, respectively). Disruption of the monolayer with gaps between endothelial cells (*), alterations in protein patterns (arrowheads) with the presence of dot-like staining (yellow arrow), and perinuclear distribution (arrows) are indicated. Representative results from one of two independent experiments are shown.

Disorganization of the monolayer together with gaps between endothelial cells was also detected in cells exposed to the neurotoxins. In rat BMEC co-cultured with astrocytes not treated with LPS or UCB (control) for 24 h, β-catenin was mainly located at the plasma membrane. Compared to mono-cultures, LPS-treated cells also showed greater expression of β-catenin but with visible large intercellular spaces. In UCB-treated cells, on the other hand, the intensity of β-catenin immunostaining in the cytoplasm was weaker and that at the cell contours was greater than in monocultured cells at the same period. In BMEC treated with LPS and UCB, β-catenin was noticeable not only at the plasma membrane but also in the cytosol including the perinuclear region. Moreover, the staining revealed intercellular gaps.

Immunostaining for TJ proteins ZO-1 and claudin-5 was performed in mono-cultures and co-cultures after 24 h of treatment with the neurotoxins (Figs. 2.8 and 2.9). In control cells, the contour line observed for ZO-1 appeared thicker in co-cultures than in mono-cultures, similarly to that observed for β-catenin. Despite the beautiful labelling obtained for claudin-5 in mono-culture control cells, the contours appeared lighter than in co-cultures. Moreover, the staining observed in the cytosol

nearly disappeared in co-cultures and, therefore, the main immunostaining was at the plasma membrane when endothelial cells were co-cultured with astrocytes, for both ZO-1 and claudin-5. In mono-cultures, cells exposed to LPS or UCB alone, or to their combination, showed altered morphology and staining patterns for ZO-1 and claudin-5, as compared with untreated cells that showed elongated shape and well delineated contours. Cells treated with UCB, or its combination with LPS, showed fragmented staining for claudin-5, suggesting an impairment of the intercellular junctions. Moreover, spaces were visible between adjacent cells, further confirming the damaged connection between cells following treatment with the insults. Unlike the simplified model, co-cultured cells treated with LPS looked more altered at this time-point. They showed large spaces between cells and less staining for ZO-1 at the membrane, with more cytoplasmic and perinuclear immunoreactivity. On the other hand, UCB seemed to have a greater effect on claudin-5 with a decrease in plasma membrane immunoreactivity.

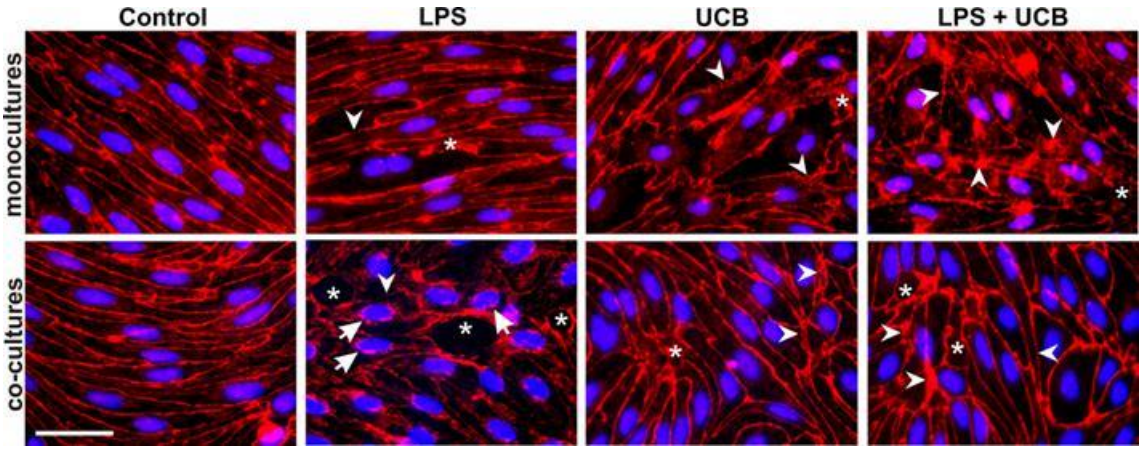


**Figure 2.8 – Lipopolysaccharide (LPS) and unconjugated bilirubin (UCB) alter *zonula occludens*-1 (ZO-1) expression in brain endothelial cells.** Cells, either in mono-culture or co-cultured with astrocytes, were fixed and immunostained with an antibody against ZO-1 to evaluate its cellular localization and pattern of expression, as well as integrity of the monolayer. Disruption of the monolayer with gaps between endothelial cells (*), alterations in protein patterns (arrowheads) and perinuclear distribution (arrows) are indicated. Representative results from one of two independent experiments are shown. Scale bar, 20 μm.

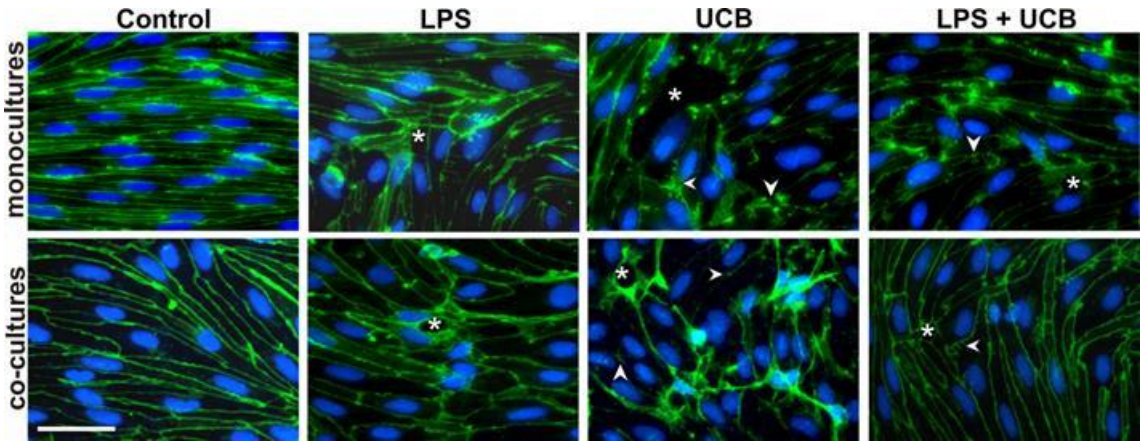


**Figure 2.9 – Lipopolysaccharide (LPS) and unconjugated bilirubin (UCB) alter expression of claudin-5 in brain endothelial cells.** Cells, either in mono-culture or co-cultured with astrocytes, were fixed and immunostained with an antibody against claudin-5 to evaluate its cellular localization, pattern of expression and integrity of the monolayer. Disruption of the monolayer with gaps between endothelial cells (*) and alterations in protein patterns (arrowheads) are indicated. Representative results from one of two independent experiments are shown. Scale bar, 20 μm.

## 4. Discussion

In the present study, confluent monolayers of primary rat BMEC were used alone or in co-culture as *in vitro* models of the BBB. The integrity of the endothelial barrier and its functional and morphological characteristics were evaluated after treatment with LPS and/or UCB. Our study shows that LDH release in primary rat BMEC is significantly modified by UCB and by both insults combined, but that at least 77% of cells are viable, which is a desirable experimental condition to perform functional studies such as the assessment of P-gp and MMPs activities. Necrotic-like cell death induced by the same concentration of LPS was reported in transformed bovine BMEC by Karahashi et al. (2009), though the extent was over 40% at 24 h and no significant damage was seen at 4 h. Such divergent viability data may not only reflect different reactions to neurotoxins between species and between cell lines and primary cultures (Silva et al. 2006), but can mostly be due to the fact that this study was performed with medium supplemented with 10% FBS, unlike our own models. In studies performed in similar conditions to ours, namely with no addition of soluble CD14, there is also little LPS-induced LDH release for up to 16 h, being this the maximum time studied (Shiori et al. 2009). Still, our conditions are suitable for LPS interaction with BMEC given that our medium contains 2% FBS, which has soluble CD14, and that EC also express membrane CD14 in the presence of LPS (Singh and Jiang 2004). LDH release induced by UCB is in agreement with our previous observations in a cell line of human BMEC (Palmela et al. 2011), although in a slightly different range. Apoptotic cell death

resulting from exposure to LPS is observed in rat primary BMEC, and the present data are in agreement with that obtained in cell lines of rat, bovine and human BMEC (Karahashi et al. 2009, Liu et al. 2004). UCB-induced apoptosis, based on analysis of nuclear morphology with Hoechst staining and confirmed by caspase-3 activity, is in line with our previous observations in a human BMEC line treated with UCB (Palmela et al. 2011), as well as with those of Akin et al. (2002) in bovine BMEC. An interesting observation from this study is that the cell damage shown by LDH release, apoptotic cell death and caspase-3 activation tend to be higher and occur earlier in UCB-treated cells than in LPS-exposed ones. Accordingly, LPS did not further enhance UCB-induced cell demise. On the other hand, while no significant apoptosis or caspase-3 activation were detected by LPS alone at 4 h, cells co-exposed to both insults at this time-point exhibited a similar profile to that of UCB.

P-gp is an important efflux transporter at the BBB, which protects the nervous system from toxic compounds. Therefore, its inhibition compromises brain protection and promotes toxicity. The retention of R123 by LPS in our experiments reflects an impairment of the transporter activity and confirms previous reports obtained both *in vitro* (Harz et al. 2006, Veszelka et al. 2007) and *in vivo* (Salkeni et al. 2009, Sequeira et al. 2007). The data collected also revealed an inhibition of P-gp activity by UCB, which is a substrate of P-gp in bovine BMEC (Sequeira et al. 2007). Brain UCB levels were increased in P-gp deficient mice (Watchko et al. 1998) and in rats treated by P-gp inhibitors (Hankø et al. 2003, Watchko et al. 1998). It is interesting to point out that ongoing studies in our laboratory revealed higher expression of P-gp in the brain parenchyma (endothelial cells, astrocytes and neurons) of a human newborn infant who died from kernicterus, the most severe pathologic outcome of hyperbilirubinemia (Brito et al. 2013). These results suggest that the increase in P-gp expression may be a compensatory mechanism to overcome the lack of efficiency in its activity at the BBB. On the other hand, if UCB levels inside endothelial cells are extremely elevated, the transporter might not have the capacity to export both UCB and R123 simultaneously, resulting in higher R123 contents.

MMPs are a family of proteases, important in normal development and in endothelial cell migration given their ability to degrade the ECM surrounding BBB capillaries (Galvéz et al. 2001). As ECM plays an important role in the induction of BBB properties (Cardoso et al. 2010), the activity pattern of secreted MMPs after exposure to LPS and/or UCB was investigated in the present study. Both MMP-2 and MMP-9 have similar activity patterns, which may be due to a homologous function. Our data are in agreement with previous studies where it has been proven that exposure to LPS results in enhanced secretion of active MMP-2 in human umbilical vascular endothelial

cells (Kim et al. 2000), and that microglial cells treated by UCB secrete active MMP-2 and MMP-9 (Silva et al. 2010). Released MMPs may lead to degradation of the ECM and weaken the barrier properties of the BBB by impairing the integrity of the basement membrane (Tilling et al. 1998), which might then favour the access of neurotoxins to the brain. Interestingly, activation of MMPs is more marked at 4 h than at 24 h, consistent with the striking alterations observed at earlier time for permeability and TEER.

Ultrastructural analysis has indicated that apoptotic cell bodies are visible following both LPS and UCB treatments, supporting our apoptosis data obtained by fluorescence microscopy. All treated cells presented disruption of the organelles, but it was interesting to notice that the impairment of ultrastructure was more marked for UCB than for LPS. This observation is consistent with the greater extent of apoptotic and necrotic-like cell death by UCB and might be related to UCB interaction with cell membranes and perturbation of their dynamic properties that we have previously demonstrated (Brito et al. 2001, Rodrigues et al. 2002). Another important observation is the enhanced formation of caveolae-like structures after LPS exposure, in line with previous observations in rats (Kittel 1999, Kittel et al. 2007). Caveolin-1 plays an important role in capillary formation and, when up-regulated, it activates MMP-2 and MMP-9 (Kim et al. 2000, Phillips and Birnby 2004) [63,69]. These MMPs were particularly amplified after exposure to LPS for 4 h, thus pointing to a possible relation between these partners.

Rat BMEC exposed to LPS still present TJs, though the TJ-stiched membrane length appeared shorter compared to untreated samples, whereas TJs are hardly visible in UCB-treated cells. This observation may suggest that paracellular passage of UCB across the endothelial monolayer may be facilitated. Although a TEM study by Böhm et al. [1995] reported UCB-induced damage of both cell and nuclear membranes in human basal skin cells, as far as we know, this is the first report describing UCB-induced ultrastructural alterations in endothelial cells forming the BBB.

Our permeability results on brain endothelial mono-cultures are in line with those recently obtained by He and collaborators (2011), where a short exposure to LPS induced hyperpermeability and TEER decrease in a BMEC line. Moreover, a study reports that TLR4 and CD14 mRNA levels increase up to 3 h after LPS exposure and then gradually decrease, though remaining detectable for up to 9 h (Singh and Jiang 2004). This observation might explain why LPS effects in mono-cultures become less marked as the incubation is prolonged. This fact, together with the higher activity of MMPs by a short exposure, shall then contribute to the greatest impairment of barrier indicators observed at 4 h. In co-cultures, the present results are supported by our

previous reports that LPS elevates permeability and decreases TEER (Veszelka et al. 2007). It has been long stated that astroglial cells strengthen BMEC barrier properties (Cardoso et al. 2010, Deli et al. 2005, Tóth et al. 2011) and prevent the increase of endothelial permeability after several stimuli, such as hypoxia or ischemia (Gesuete et al. 2011, Kondo et al. 1996). The protective effect of glial cells against LPS-mediated injury was also demonstrated in bovine brain endothelial cells (Descamps et al. 2003). Nonetheless, it has been proven that, in inflammatory conditions, astrocytes increase human BMEC permeability via IL-1β secreted to the cell culture supernatant and this could be reversed through a monoclonal antibody against IL-1β (Didier et al. 2003). Regarding UCB, no former studies have been performed on its effects on brain endothelial barrier properties. Raimondi et al. (2006) demonstrated that exposure of human intestinal cell line Caco-2 to UCB decreases transepithelial electrical resistance and increases the paracellular flux of 10 kDa dextran. Our present findings obtained in rat BMEC regarding UCB-induced decrease in TEER and increase in permeability are consistent with the data found in Caco-2 epithelial cells (Raimondi et al. 2006). Unlike the intestinal cells, rat BMEC monolayers do not recover at later time-points, indicating high endothelial sensitivity to UCB toxicity and a greater potential of UCB for neurotoxicity. Endothelial cells treated with both compounds for 24 h displayed more serious barrier dysfunction as compared with LPS alone, pointing to a failure of the recovery process that might happen when sepsis occurs simultaneously with hyperbilirubinemia. IL-6 is released by human BMEC, which additionally produce nitric oxide (Palmela et al. 2011), a molecule that contributes to enhance the permeability of the barrier (Cardoso et al. 2010). Rat BMEC produce prostacyclin, as we have previously demonstrated (Kis et al. 1999). Thus, changes in prostanoid, especially prostacyclin synthesis (Bachschmid et al. 2003) may also contribute to the observed endothelial permeability alterations. Interestingly, an increased permeability and a decreased TEER were also observed when each of the neurotoxins, or their combination, were added to BMEC in co-culture with astrocytes. This observation, relying in an *in vitro* model that more closely resembles the *in vivo* condition, reinforces the data obtained in the simpler model of the BBB composed of BMEC alone.

Analysis of the AJ protein distribution pattern revealed a decreased localization at intercellular junctions at 4 h exposure to LPS, UCB or their combination, which is partially reversed at 24 h, as well as a disorganization of the endothelial sheet, particularly evident in co-cultured cells. Also interesting was the perinuclear accumulation of β-catenin, which was associated with a disruption of the VE-cadherin/β-catenin complexes during inflammation (Barbieri and Weksler 2007, Ding and Yuan 2007). Therefore, the production of cytokines by astrocytes and BMEC

exposed to LPS or UCB (Fernandes et al. 2004, Fernandes et al. 2006, Palmela et al. 2011, Verma et al. 2006) may contribute to this process, which brings new insights into the mechanisms of toxicity by these compounds. Since *Wnt* signalling pathway activation is recognized as a mechanism of cell survival involved in the expression of TJ-associated proteins (Liebner et al. 2008), the present observations suggest an attempt to compensate the loss of integrity resulting from exposure to the insults.

The expression and localization of the TJ proteins ZO-1 and claudin-5 are also affected by the tested neurotoxins. The accumulation of these proteins in the plasma membrane of co-cultured control cells, compared to mono-cultured cells is in line with the astrocytic induction of barrier properties in endothelial cells *in vitro* (Cardoso et al. 2010, Deli et al. 2005, Tóth et al. 2011). It may also be related with the low serum concentration used in this study, as astrocyte co-cultures in the absence of serum in the basolateral compartment result in higher BMEC barrier indices, given their greater resemblance to the *in vivo* microenvironment (Colgan et al. 2008). Our mono-culture results confirm that LPS treatment alters intercellular junctions that are associated with BBB impairment (Singh et al. 2007). UCB-treated cells also show a redistribution of TJs proteins similarly to what was seen for occludin in epithelial cells (Raimondi et al. 2006). The segmentation of claudin-5 immunostaining pattern may be related to the secretion of active MMPs, as it was described for occludin (Reijerkerk et al. 2006, Zeissig et al. 2004). Considering that Yang et al. (2007) have reported a reduction of claudin-5 mRNA in mice following an increase in MMP-2 expression, it is conceivable that activation of MMPs might be related to the loss of claudin-5. To the impairment of TJs and consequent increase in permeability may also account nitric oxide (Wu et al. 2011), which is produced by exposure of BMEC and glial cells to UCB or LPS (Vezselka et al. 2007, Palmela et al. 2011, Silva et al. 2011).

The increase in the permeability of co-cultured rat BMEC exposed to the neurotoxins might be related to the alterations in the pattern of expression and cellular localization of ZO-1 and claudin-5, already demonstrated at earlier time-points (Veszelka et al. 2007). In fact, the mRNA contents of TJ proteins ZO-1 and claudin-5, as well as their cellular localization decrease in bovine retinal endothelial cells treated by IL-1β and TNF-α (Aveleira et al. 2010), which are known to be secreted by astrocytes. The translocation of ZO-1 from the membrane to the nucleus has never been reported in endothelial cells treated with LPS. Still, a recent paper by Zhong and collaborators (Zhong et al. 2012) describes that ZO-1 is present in small concentrations in the nuclei, even in untreated cells. Therefore, our results are in agreement with the dual role of ZO proteins (Bauer et al. 2010), and the evident increase of the perinuclear staining brings new insights into a possible translocation of ZO-1 to the nucleus or

acting as a nuclear factor during inflammation in the presence of astrocytes. Its dissociation from the junctional complexes is related with increased barrier permeability (Choi and Kim 2008), which may further explain the mechanism for enhanced permeability in co-cultures. The same permeability enhancement in UCB-treated cells can also be related to the redistribution of claudin-5, an important contributor to TJs seal (Cardoso et al 2010).

## 5. Conclusions

The present data prove that LPS and UCB alter various aspects in rat BMEC. We can conclude that these neurotoxins diminish the viability of brain endothelial cells and alter BBB functions by inhibiting P-gp activity and inducing the secretion of active MMPs. Ultrastructural analysis detected newly formed caveolae-like structures and damage of organelles, like mitochondria and rough endoplasmic reticulum, which may be involved in the mechanisms of action of these neurotoxins. Moreover, this is the first study to demonstrate that paracellular permeability, TEER and intercellular junctions are altered by these compounds in rat BMEC either in mono-cultures or in co-culture with astrocytes. At last, our study revealed that each of the compounds has its own mechanism of action and temporal profile, comprising cell death (early occurring for UCB and only lately for LPS), the impairment of barrier properties (sustained along time for UCB but not for LPS), the distribution pattern of intercellular junctions as β-catenin (opposite variations in fluorescence intensity for UCB and LPS) and ultrastructural alterations (shortened intercellular junctions and the increased number of caveolae particularly evident in LPS-treated cells). Collectively, by compromising the BBB integrity, the alterations resulting from exposure to LPS and/ or UCB may facilitate the access of the compounds to the brain further contributing to their neurotoxicity.

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*Chapter 3*

# SYSTEMIC INFLAMMATION IN EARLY NEONATAL MICE INDUCES TRANSIENT AND LASTING NEURODEGENERATIVE EFFECTS

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## Abstract

The inflammatory mediator lipopolysaccharide (LPS) has been shown to induce acute gliosis in neonatal mice. However, the progressive effects on the murine neurodevelopmental program over the week that follows systemic inflammation are not known. Thus, we aimed to investigate the effects induced by daily systemic LPS-administration from postnatal days 4 through 6 on the developing CNS. LPS administration led to acute body and brain weight loss as well as overt structural changes in the brain such as cerebellar hypoplasia, neuronal loss/shrinkage, and delayed myelination. The delay in myelination was linked to impaired proliferation or differentiation of NG2 progenitor cells at early time points following LPS administration rather than excessive phagocytosis by CNS myeloid cells. In addition to disruptions in brain architecture, evidence of a robust inflammatory response to LPS was observed. Quantification of inflammatory biomarkers revealed decreased expression of autotaxin with concurrent increases in HMGB1, TLR-4, and MMP-9 expression. This was associated with acute astrogliosis ($GFAP^+$ cells) in the brain parenchyma and at the microvasculature interface, as well as microgliosis ($CX3CR1^+$ cells) in the parenchyma. This was followed by migration/proliferation of $CX3CR1^+$ cells around the vessels at later time points, and posterior loss of $GFAP^+$ astrocytes that did not appear to result from apoptotic cell death. Collectively, our study has uncovered a complex innate inflammatory reaction and associated structural changes in the brains of neonatal mice challenged peripherally with LPS. These findings may explain some of the neurobehavioral abnormalities that develop following neonatal sepsis.

## 1. Introduction

Sepsis is a common severe inflammatory response to an infection and is associated with high infant mortality rate during the pre-natal and neonatal periods. Survivors frequently experience white matter damage and cerebral palsy as well as cognitive and affective disorders (Hall et al. 2011; Paris et al. 2011). As it is usually induced *via* bacteria that express lipopolysaccharide (LPS), sepsis is often modelled in rodents using intraperitoneal injections of this endotoxin (Buras et al. 2005).

Brain uptake of systemic LPS is considerably low (Banks and Erickson 2010), so most of its effects are mediated by brain microvascular endothelial cells (BMEC) and the activation of downstream signaling pathways (Grab et al. 2011). BMEC physically separate the brain from the blood, forming the basis of the blood-brain barrier (BBB). These cells express the Toll-like receptor (TLR)-4, whose activation by LPS leads to the synthesis and release of pro-inflammatory cytokines (Cardoso et al. 2010; Nayak et al. 2014). In addition, LPS triggers the release of activated matrix metalloproteinase (MMP)-9 and MMP-2 as well as of granulocyte-macrophage colony-stimulating factor (GM-CSF), which may contribute to the infiltration of monocytes and to BBB breakdown (Cardoso et al. 2012; Dohgu et al. 2011). BMEC are part of the neurovascular unit (NVU) that also includes the basement membrane, astrocytic endfeet, pericytes, microglia, neurons and even oligodendrocytes (Sá-Pereira et al. 2012). The direct response of these cell types to inflammatory stimuli or to the released pro-inflammatory signals may exacerbate the damaging effects on BMEC (Grab et al. 2011; Tripathi et al. 2009).

Astrocytes are essential for synapse formation, induction of BBB functions and protection against LPS-mediated injury (Daneman et al. 2010; Descamps et al. 2003; Siddharthan et al. 2007). However, in the rodent central nervous system (CNS), astrogenesis occurs late in neurodevelopment and the microvessel ensheathment by their endfeet begins only around postnatal day (PND)5 (Daneman et al. 2010). In addition, LPS triggers astrocytic production of pro-inflammatory cytokines, particularly in immature cells (Falcão et al. 2005), directly influencing neurodegeneration (Falcão et al. 2014). LPS also induces the resident immune cells of the brain, microglia, to release pro-inflammatory cytokines and other inflammatory mediators like the high-mobility group box 1 (HMGB1) protein (Lamar et al. 2011) and autotaxin (ATX) (Awada et al. 2012) that mediate changes in neuronal network activity and apoptosis (Nimmervoll et al. 2013). Though microglia progenitors colonize the mouse brain early in embryogenesis, the main transition from amoeboid into a ramified shape occurs during the second week after birth, along with increased microglial numbers and

simultaneously with the maturation of neurons (Harry and Kraft 2012; Nayak et al. 2014). The morphological features of microglia and their colonization of the mouse brain are similar in humans (Torres-Platas et al. 2014).

Acute (first hours) and prolonged sepsis (days rather than hours) have been shown to manifest differently. A robust inflammatory response is mounted during the acute phase, which to some degree is counterbalanced by an anti-inflammatory response during the later stages (Albert et al. 2003). Despite induction of an anti-inflammatory program, studies have shown that prolonged injection of LPS can potentiate proinflammatory cytokine levels in the brain (Xiao et al. 2006). It is therefore important to understand the pathogenesis of sepsis when proinflammatory cytokine levels are sustained in the developing neonatal brain for extended time periods. A better understanding of the sepsis-induced CNS pathogenesis will provide an important first step in improving survival and preventing adverse outcomes. In this study we aimed to investigate the acute and lasting effects of LPS-administration on murine neurodevelopment during the first week of life. We found that prolonged systemic inflammation disrupts the immature CNS by causing neuronal atrophy, a delay in myelination and acute reactive gliosis.

## 2. Materials and Methods

### 2.1. Animals

Pregnant CD1 *wild-type* (WT) mice at embryonic day (E)16 were purchased from Harlan Ibérica (Spain) laboratories and gave birth in the animal facility of the Faculdade de Farmácia, Universidade de Lisboa. To better explore microglia activation, we used heterozygous C57BL/6 (B6) CX3CR1$^{gfp/+}$ mice. These mice were generated by crossing B6 WT with B6 CX3CR1$^{gfp/gfp}$ mice that were purchased from The Jackson Laboratory (USA) and maintained in a closed breeding facility at The National Institutes of Health (NIH), Bethesda. The insertion of the green fluorescent protein (GFP) allows the tracking of CX3CR1$^{+}$ cells, which is important to visualize microglia dynamic changes (Huang et al. 2006; Nayak et al. 2012). Homozygous B6 CX3CR1$^{gfp/gfp}$ mice are CX3CR1 deficient and do not respond to fractalkine. On the other hand, both B6 WT and CX3CR1$^{gfp/+}$ mice respond similarly to LPS (Jung et al. 2000).

Mice were housed with a 12 hour light/dark cycle and were provided with *ad libidium* access to a standard laboratory chow diet and drinking water. This study was carried out in strict accordance with the recommendations of European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific

Purposes (Council Directive 86/609/EEC), as well as with those in the Guide for the Care and Use of Laboratory Animals at the NIH. All experimental procedures were performed under anesthesia, conducted in a manner to minimize animal suffering and all efforts were made to use the minimum number of animals.

### 2.2. Drug Administration

The day of birth was defined as PND1. For each strain, offspring of both genders were randomly divided into two groups and were treated from PND4 to PND6 with daily intraperitoneal injections of either endotoxin-free saline [control (W/O LPS); n ≥ 4 per analysis] or of LPS [6 mg/kg, Escherichia coli 055:B5; Calbiochem (Merck, Germany); n ≥ 4 per analysis] to induce - systemic inflammation (Erickson et al. 2012; Jin et al. 2011). CD1 WT mice were sacrificed 1 day after the final LPS administration (LPS1) and at LPS9 to evaluate acute and lasting effects, respectively. B6 CX3CR1$^{gfp/+}$ mice were sacrificed at LPS1/3/5/6/7/9 not only to determine the acute and lasting effects but also the delayed effects. Injection and sampling regimens are depicted in Figure 3.1.

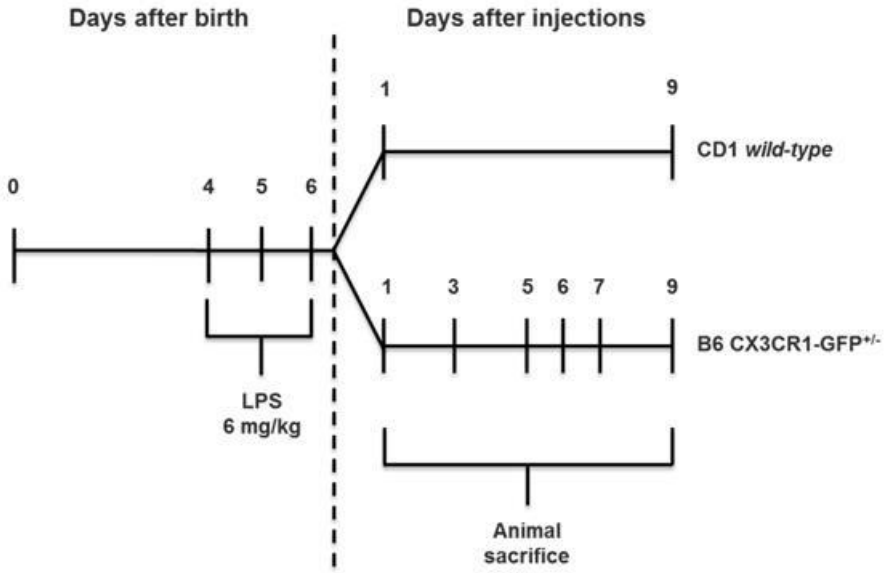



**Figure 3.1 – Schematic representation of the early induction of systemic inflammation.** Offspring of both genders were randomly divided into two groups and treated with three intraperitoneal injections of either saline solution or lipopolysaccharide (LPS) at days 4, 5 and 6 after birth to induce systemic inflammation. CD1 *wild-type* mice were sacrificed at 1 and 9 days after LPS injections, and C57BL/6 (B6) CX3CR1$^{gfp/+}$ mice were sacrificed at days 1/3/5/6/7/9 following LPS administration.

### 2.3. Tissue Process

For paraffin-histological analysis, CD1 WT mice were perfused *via* the ascending aorta with 4% paraformaldehyde (PFA) in PBS. Brains were post-fixed in the indicated fixative for at least 24 h. Brain tissue was processed for paraffin and cut into 6 µm sagittal sections. For gelatin zymography and western blot analysis, the same animals were perfused *via* the ascending aorta with phosphate buffer saline solution, pH 7.4 (PBS). Brains were quickly removed, snap-frozen and cryopreserved at -80ºC for at least 24 h. Protein extracts were obtained by lysing the brain tissue with radioimmunoprecipitation assay (RIPA) buffer (Tris Buffer 1 M pH 8.0, EDTA 0.5 M pH 8.0, NaCl 5 M, 10% NP-40, 50% glycerol, 10% SDS) (Ratilal et al. 2014). For cryo-histological analysis, B6 CX3CR1$^{gfp/+}$ animals were perfused with 4% PFA in PBS. Brains were post-fixed in the same fixative for 24 h, followed by 15% and 30% sucrose solutions, each for at least 16h. Brain tissue was embedded in TFM$^{TM}$ Tissue Freezing Medium (TBS® Triangle Biomedical Sciences, USA), frozen at -80ºC and cut with a cryomicrotome into 25 µm sagittal sections.

### 2.4. Staining for Luxol Fast Blue and Cresyl Violet

Paraffin sections were stained with Luxol Fast Blue (VWR, USA) for oligodendrocyte myelin followed by with Cresyl Violet (Sigma, USA) for neuronal Nissl bodies' assessment. Sections were rehydrated in xylene for 20 min and decreasing concentrations of ethyl alcohol for 10 min each. Sections were then incubated with a 0.1% Luxol Fast Blue solution in 70% ethyl alcohol overnight at 56ºC. After rinse with 70% ethyl alcohol followed by distilled water to remove excess stain, the tissue was differentiated in a 0.5% lithium carbonate solution. Slides were then rinsed in distilled water followed by 2% acetic acid solution for 5 min. Next, tissue was counterstained with a 1% Cresyl Violet solution for 10 min. After rinse in distilled water, sections were differentiated in 37.5% acetic acid and rinsed in distilled water. Slides were mounted with Fluoromount-G (Southern Biotech, USA) and visualized using a DFC 490 camera (Leica, Germany) adapted to an Axioskop bright field microscope (Zeiss, Germany). Width of cerebellar layers, number of neurons per field and the area of their soma were analyzed using ImageJ software (NIH, USA). Intensity of staining of the external germinal layer at LPS1 and of the white matter layer at LPS9 was analyzed using the same software and was expressed per µm$^2$.

### 2.5. Gelatin Zymography

Determination of MMP-9 and MMP-2 was evaluated as previously described (Cardoso et al. 2012) with minor alterations. In short, 40 µg of protein from tissue

extracts were analyzed by SDS-PAGE zymography in 0.1% gelatin/10% acrylamide gels under non-reducing conditions. After electrophoresis, gels were washed for 1 h with 2.5% Triton X-100 (in 50 mM Tris pH 7.4, 5 mM $CaCl_2$, 1 mM $ZnCl_2$) to remove SDS and renature the MMPs species in the gel. Gels were then incubated in developing buffer (50 mM Tris pH 7.4, 5 mM $CaCl_2$, 1 mM $ZnCl_2$) for 72 h at 37ºC to induce gelatin lysis. For enzyme activity analysis, gels were stained with 0.5% Coomassie Brilliant Blue R-250 (Bio-Rad, USA) for 3 h at room temperature (RT), and distained in 30% ethanol/10% acetic acid/$H_2O$. Gelatinase activity, detected as a white band on a blue background, was quantified by computerized image analysis using Quantity One 1-D Analysis Software (Bio-Rad, USA).

### 2.6. Western Blot

Western blot analysis was performed as previously described (Ratilal et al. 2014) with minor alterations. Briefly, 100 µg of protein from tissue extracts were separated on a 12% SDS-PAGE gel. Following electrophoretic transfer onto a nitrocellulose membrane and blocking with 5% milk solution, the blots were incubated with primary antibody overnight at 4ºC [rabbit anti-TLR4 (Santa Cruz, USA, #sc-10741; 1:500), mouse anti-HMGB1 (BioLegend, USA, #651402; 1:500), rabbit anti-ATX (Millipore, USA, #ABT28; 1:500) or mouse anti-β-actin (Sigma, USA, #A5441; 1:5000)] and with horseradish peroxidase-labeled secondary antibody [anti-mouse or anti-rabbit (Santa Cruz, USA, #sc-2005 and #sc-2004 respectively; 1:5000] for 1 h at RT. Protein bands were detected by LumiGLO® (Cell Signaling, USA) and visualized by chemiluminescence with ChemiDoc (Bio-Rad, USA). Expression was quantified by computerized image analysis using Quantity One 1-D Analysis Software (Bio-Rad, USA).

### 2.7. Cryo-Immunofluorescence

Frozen sections were used to analyze the expression of the fractalkine receptor, CX3CR1, as well as $NG2^+$ glia, GFAP, myelin basic protein (MBP) and CD31 in B6 $CX3CR1^{gfp/+}$ mice. Sections were fixed for 15 min with 1% PFA in PBS. Sections were then treated with an Avidin/Biotin Blocking Kit (Vector Laboratories, USA, #SP-2001) per the manufacturer's instructions followed by 20 min treatment with Background Buster (INNOVEX biosciences, USA, #NB306). Tissue was incubated 1 h at RT with primary antibodies: rabbit anti-NG2 (Millipore, USA, #AB5320; 1:100), rabbit anti-GFAP (DAKO, Denmark #Z0334; 1:200), rat anti-MBP (Millipore, USA, #MAB386; 1:100), or armenian hamster anti-CD31, clone 2H8 (Chemicon, USA, #MAB1398Z; 1:200). Following the incubation with primary antibodies, sections were washed and incubated

for 1h at RT with secondary antibodies donkey Alexa Fluor 647 anti-rabbit IgG (H&L) (Jackson ImmunoResearch Laboratories, USA, #711-605-152; 1:200), donkey Alexa Fluor 647 F(ab) anti-rat IgG (H&L) (Jackson ImmunoResearch Laboratories, USA, #712-606-150; 1:200) or goat Rhod-X anti-armenian hamster IgG (H&L) (Jackson ImmunoResearch Laboratories, USA, #127-295-160; 1:200). CD31 staining was amplified with donkey Rhod-X F(ab) anti-goat IgG (H&L) (Jackson ImmunoResearch Laboratories, USA, #705-296-147; 1:200) for 1 h at RT. All working stocks of primary and secondary reagents were diluted in PBS containing 2% fetal bovine serum (FBS) + 0.5% Triton-X100. Nuclei were counterstained with DAPI dye, and sections were mounted with IMMU-MOUNT (Thermo-Scientific, USA, #9990402). Between incubations, sections were washed three times with PBS. Apoptosis was detected in frozen sections with the ApopTag® Red *In Situ* Apoptosis Detection Kit (Chemicon, USA, #S7165), which specifically detects DNA cleavage and chromatin condensation associated with apoptosis, in accordance with the manufacturer's instructions. Images were captured from stained frozen sections using an Olympus FV1200 confocal microscope equipped with 20× and 40× objectives. Images were collected using sequential scanning with the 405, 488, 559, and 635 nm laser lines to produce four color overlays. Cerebellar area was measured in tiles of DAPI-counterstained brain sections using ImageJ (NIH, USA). Area fraction and colocalization of the staining per field of each protein was quantified by computerized image analysis using ImageJ (NIH, USA).

### 2.8. Sholl Analysis of CX3CR1$^{+}$ cells

To quantify morphological changes of the CX3CR1$^{+}$ cells, consecutive Z-stack Images were converted to a maximum intensity projection image by Image J software. Using the Sholl analysis plugin, concentric circles were created centered on the soma, beginning at 5.5 µm radii and increasing 2 µm with every circle. We determined the number of intersections made by microglia branching processes with each successive increasing circle, the maximum number of intersections for the cell (Nm), as well as the critical value at which Nm occurred (Cr) and the maximum length at which a branch intersection occurred (Morrison and Filosa 2013).

### 2.9. Statistical Analysis

Results are expressed as means ± SEM from, at least, four independent animals in each treatment group. Significant differences between groups were determined by the two-tailed t-test performed on the basis of equal and unequal variance as appropriate. Comparison of more than two groups was done by ANOVA using

GraphPad Prism® 5.0 (GraphPad Software, San Diego, CA, USA). Statistical significance was considered when *P* values were lower than 0.05.

## 3. Results

### 3.1. LPS administration in the early neonatal period triggers acute weight loss and cerebellar hypoplasia

Administration of LPS is known to induce a sickness behavior in adult mice, including weight loss (Lawson et al. 2013; Walker et al. 2013). To assess the impact of LPS exposure in the first postnatal week, we initially assessed body and brain weight oscillations, which were acutely decreased by LPS (Fig. 3.2A). Body weight loss was sustained up to LPS7, but eventually recovered by LPS9 (Fig. 3.2B). We further explored acute brain weight loss by measuring the sagittal cerebellar area. This revealed a disruption in its development evidenced by a ~2-fold reduction at LPS5 that was still evident at LPS7. No difference from the control group was observed at LPS9 (Fig. 3.2C,D).

### 3.2. Induction of neuronal atrophy in newborn mice by systemic inflammation is more marked at LPS1 than at LPS9

Given the observed decrease in the cerebellar area, we next explored the width of each layer: the external germinal layer (with proliferating neuroepithelial cells), the molecular layer (containing the axons of granule cells and dendrites of Purkinje cells), the Purkinje neuronal layer, the granular layer (with small neurons called granule cells) and the white matter layer (with myelin fibers) (representative images of cerebellum are shown in Figure 3.3A). There was a significant shrinkage of the neuron-containing layers (external germinal, Purkinje and granular) at 24 h after the last LPS injection but not at LPS9 (Fig. 3.3B). In agreement, the density of neurons based on the intensity of staining per $\mu m^2$ of cells in the external germinal layer was also markedly reduced at LPS1 (~2-fold) (Fig. 3.3C). Lastly, a negative impact on the density of Luxol Fast Blue-labeled myelin fibers in the cerebellum was evident at LPS9 (Fig. 3.3D).

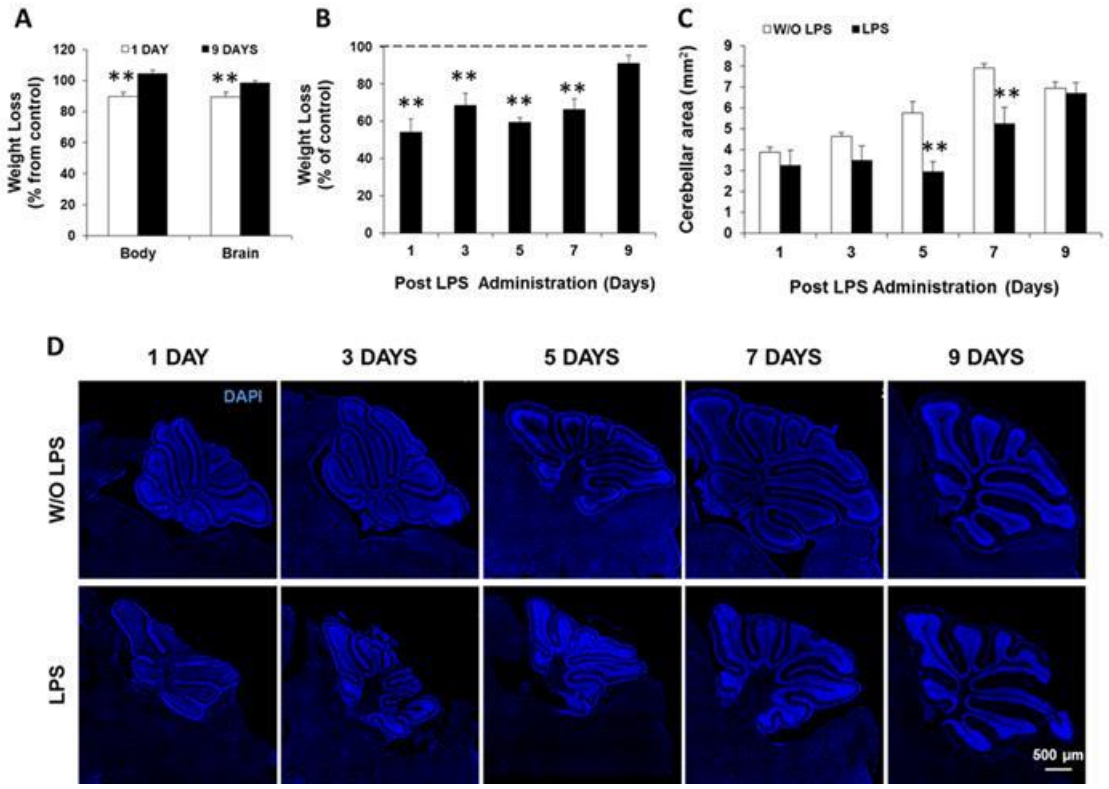



**Figure 3.2 – Lipopolysaccharide (LPS) administration in the early neonatal period leads to an acute, but transient, body and brain weight loss as well as to a decreased cerebellar area.** Body and brain of CD1 *wild-type* mice were weighed at 1 and 9 days post-LPS administration (A). Body weight of C57BL/6 (B6) CX3CR1$^{gfp/+}$ mice was assessed at days 1/3/5/7/9 after LPS injection (B). Cerebellar area was measured (C) in tiled confocal images of brain sections from B6 CX3CR1$^{gfp/+}$ mice at days 1/3/5/7/9 post-LPS (representative images with DAPI in D). Results are mean ± SEM from at least four animals. **P < 0.01 *vs*. without (W/O) LPS.

We next searched for changes in neuronal density and morphology by examining brain regions such as in pons [mediator of cerebellar input and output (Baizer 2014)], the CA3 subregion of the hippocampus [responsible for short-term memory (Kesner 2007)] and the Purkinje cell layer that provides the output of all motor coordination in the cerebellar cortex (Kullmann et al. 2012) (representative images are showed in Figure 3.3A). The number of neurons was acutely decreased at LPS1, particularly in pons, where it was still evident at LPS9. The neuronal loss in both hippocampus and cerebellum seen acutely at LPS1 was restored by LPS9 (Fig. 3.3E). On the other hand, the marked soma reduction observed in neurons at LPS1 persisted until LPS9 in all three regions (Fig. 3.3F).

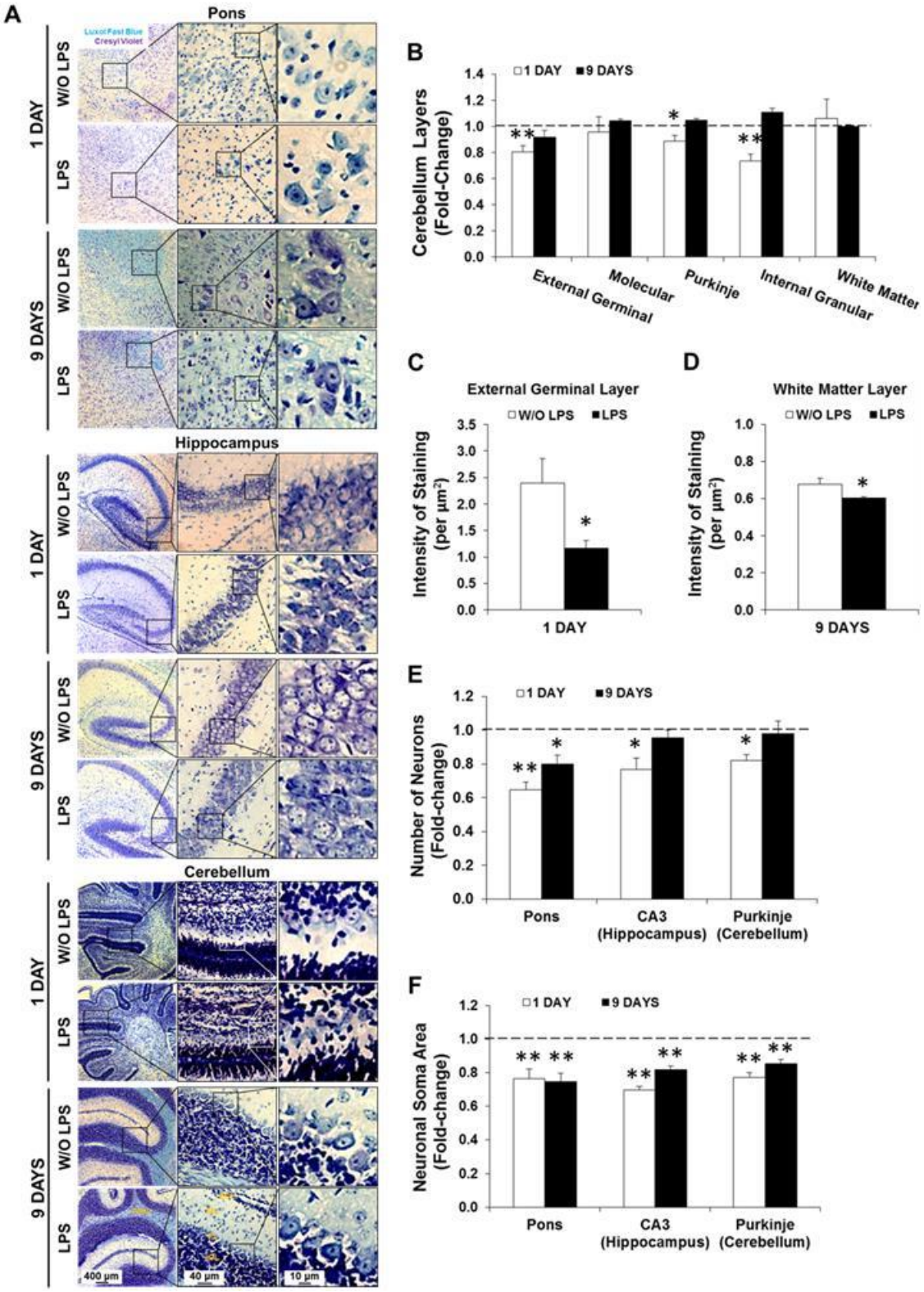

A
Pons
Luxol Fast Blue
Cresyl Violet
1 DAY
9 DAYS
W/O LPS
LPS
Hippocampus
Cerebellum
400 µm
40 µm
10 µm
B
1 DAY
9 DAYS
Cerebellum Layers (Fold-Change)
External Germinal
Molecular
Purkinje
Internal Granular
White Matter
C
External Germinal Layer
W/O LPS
LPS
Intensity of Staining (per µm²)
1 DAY
D
White Matter Layer
9 DAYS
E
Number of Neurons (Fold-change)
Pons
CA3 (Hippocampus)
Purkinje (Cerebellum)
F
Neuronal Soma Area (Fold-change)

**Figure 3.3 – Early lipopolysaccharide (LPS) administration triggers acute shrinkage of cerebellar layers and loss of neuronal density as well as a sustained reduction of neuronal soma.** Paraffin sections from CD1 *wild-type* mice at 1 and 9 days post-LPS administration were stained with Luxol Fast Blue (myelin, blue) followed by Cresyl Violet (Nissl bodies, purple). Representative images are shown for each condition in pons, hippocampus and cerebellum (A). The widths of each cerebellar layer [external germinal layer (EGL), molecular layer (ML), Purkinje layer, internal granular layer (IGL), white matter layer (WML)] were measured (B). Intensity of Cresyl violet staining in the EGL (C) and of Luxol Fast Blue in WML (D) were quantified per $\mu m^2$ at 1 and 9 days after LPS administration, respectively. The number of neurons per field (E) and the area of neuronal cell body (soma) (F) was quantified throughout pons, in the CA3 hippocampal region and in the cerebellar Purkinje layer (PL). All determinations were done using ImageJ software (NIH, USA). Results are mean ± SEM from at least five animals. *P < 0.05 *vs*. without (W/O) LPS.

### 3.3. LPS administration leads to a reduced myelination

Given the significant impact on the myelin layer and the amount of neuronal damage evident in the pons and cerebellum, we further analyzed the effects of LPS on myelination in these two brain regions at days LPS1,3,5,7 and 9 (representative images in Fig. 3.4A). Reduced levels of MBP per unit area were observed at all time points, but the decreases were particularly evident at LPS5 (minimum values) and LPS9 in both brain regions when compared to controls (Fig. 3.4B). Considering the significant reduction of MBP at LPS5, we decided to investigate the contribution of $CX3CR1^+$ microglia to this abnormality. We hypothesized that their phagocytotic activity might be associated with the delayed myelination. However, as shown in Figure 3.4C, this was not the case, as microglia did not contain cytoplasmic myelin signal at LPS1 or LPS3. To determine whether apoptosis of oligodendrocyte precursors cells (OPCs) could contribute to the decreased myelination, we stained tissues at LPS1 with ApopTag and anti-NG2 antibodies (expressed by OPCs) (Fig. 3.4D). Increased apoptosis was observed in the cerebellum (particularly in the external germinal layer) as well as in the pons; however, no overlap of $NG2^+$ labeling with ApopTag was noticed. Moreover, we observed an increased number of OPCs in the cerebellum (4-fold) and pons (1.4-fold) (Fig. 3.4E). These data suggest that there may be an increased proliferation of OPCs, or, alternatively, a delay in their maturation to myelinating cells resulting from neuroinflammation.

### 3.4. Early neonatal LPS administration acutely decreases ATX levels while increasing other inflammatory biomarkers

We next explored the neuroinflammatory reaction to systemic LPS injection by quantifying the expression of inflammatory biomarkers in whole brain tissue. The biomarkers MMP-9, MMP-2, TLR4, HMGB1 and ATX are known to be expressed by astrocytes, microglia, and / or BMECs during inflammatory conditions. We therefore

evaluated if they were affected by the LPS administration and, if so, whether the effects were lasting (Fig. 3.5). With the exception of MMP-2, all biomarkers were acutely modified by LPS administration. MMP-9 expression was modestly increased at LPS1, whereas TLR4 and HMGB1 levels were elevated more significantly. ATX expression, on the other hand, was significantly decreased. The results indicate that the pro-inflammatory response observed 24 hours after LPS administration was not sustained, as all biomarkers were restored to control levels at LPS9.

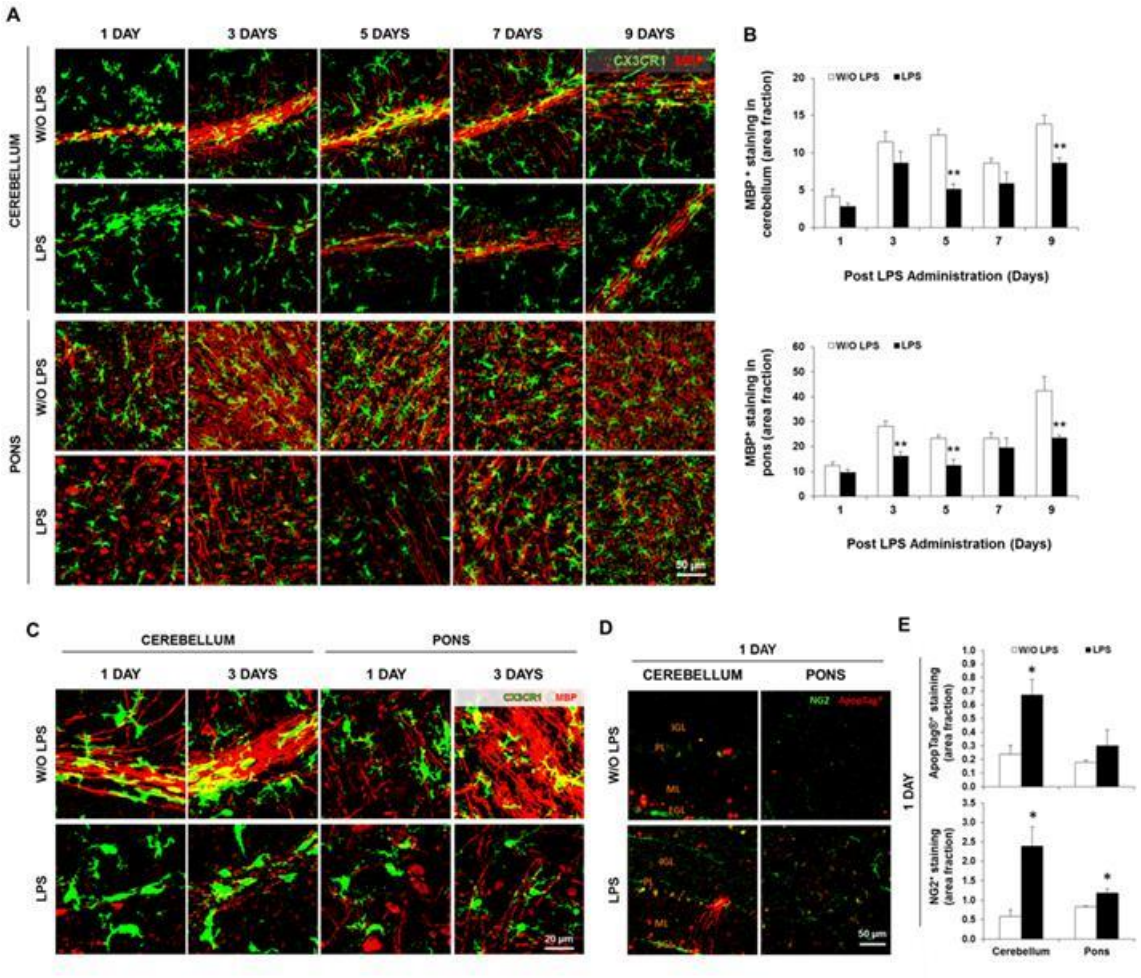



**Figure 3.4 – Lipopolysaccharide (LPS) administration disrupts myelination in the cerebellum and pons of neonatal mice.** Brain cryosections from C57BL/6 CX3CR1$^{gfp/+}$ mice at days 1/3/5/7/9 post-LPS administration were immunolabeled for myelin (myelin basic protein, MBP, red). Representative confocal images of cerebellum and pons are displayed in A, where the overlapping of MBP and microglial marker CX3CR1 (green) is seen in yellow. Area fraction per field of MBP staining was quantified per region (B) using ImageJ software (NIH, USA). To assess microglial phagocytosis, confocal images at days LPS1 and LPS3 were amplified (C, overlapping of MBP and CX3CR1 is visible in yellow). Sections of animals at LPS1 were immunolabeled for apoptosis (ApopTag, red) and oligodendrocytes precursors (NG2 cells, green). Cerebellar layers are identified [external germinal layer (EGL), molecular layer (ML), Purkinje layer, internal granular layer (IGL)] (D). Area fraction per field of ApopTag and of NG2 positive stainings were determined (E) using ImageJ software (NIH, USA). *P < 0.05 and **P < 0.01 *vs*. without (W/O) LPS.

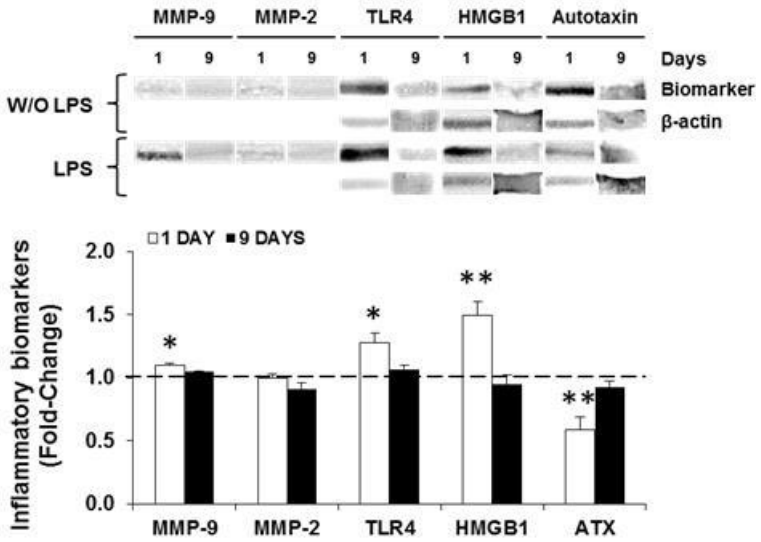



**Figure 3.5 – Lipopolysaccharide (LPS) challenge promotes differential expression of inflammatory biomarkers in the brain.** Whole brain lysates of CD1 *wild-type* mice at 1 day post-lipopolysaccharide (LPS) administration were used to determine the activities of metalloproteinase(MMP)-9 and MMP-2 by gelatin zymography, alongside with the expression of Toll-like receptor (TLR)-4, high-mobility group box 1 (HMGB1) and autotaxin (ATX) by western blot. Results are expressed as fold-change from animals not treated with (W/O) LPS, and are mean ± SEM from at least four animals in each group. *P < 0.05 and **P < 0.01 *vs*. W/O LPS.

## 3.5. Neonatal inflammation decreases the microglia transition from an amoeboid to ramified morphology

The alterations in inflammatory biomarkers suggested that microglia might be involved in the response to peripheral LPS challenge. Therefore, we decided to examine microglial morphology following neonatal LPS administration in the pons – the brain region most affected by this endotoxin. Analysis of parenchymal $CX3CR1^{gfp/+}$ cells in control mice revealed that microglia gradually changed morphology from an amoeboid phenotype at the end of the first week of life to a more ramified morphology by the second week (Fig. 3.6A). This is supported by our quantitative data showing that the maximum number of microglia process interactions (process maximum, Nm), and the distance from the soma where these interactions occurred (critical value), increased over time in control mice (Fig. 3.6B,C). However, these parameters were significantly decreased by LPS treatment. In fact, at LPS1 and LPS3 $CX3CR1^{+}$ cells were still amoeboid. By LPS5, microglia showed elongated soma and few secondary processes, which progressed to further enlargement of the soma and a reduced number of short processes at LPS7 and LPS9 (Fig. 3.6A). Quantitatively, at LPS9 the maximum radius at which a branch intersection occurred (maximum branch length) was significantly reduced (25%). However, no alterations in the number of branches

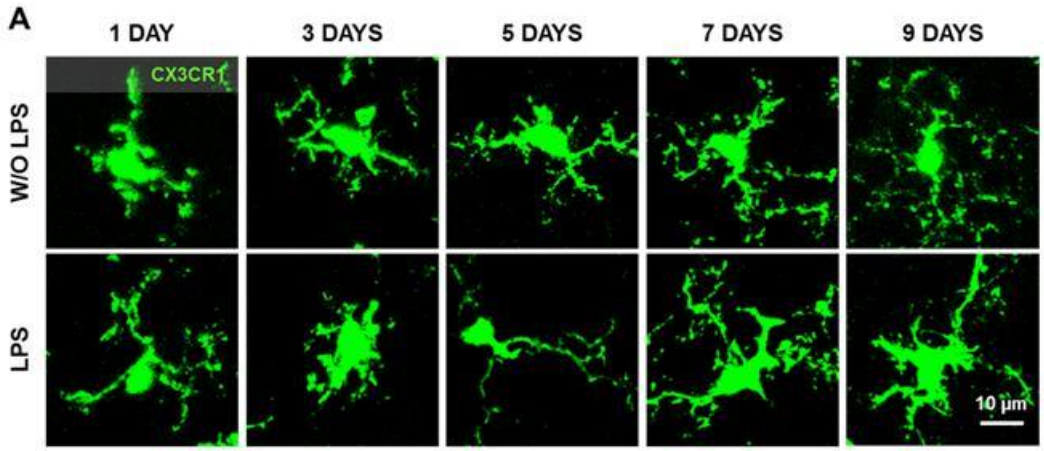
that originated from microglia soma (number of primary branches) or in the cell branching density (Schoenen ramification index) were noticed (Fig. 3.6B,C).

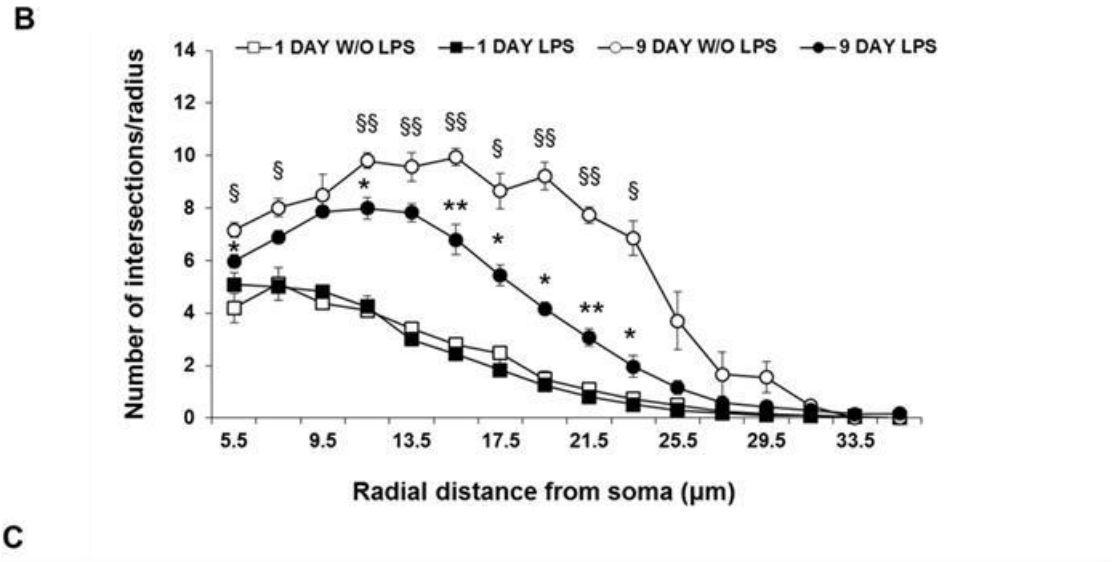
### 3.6. Decreased astrocytosis occurs after LPS5 and is inversely associated with microgliosis

To further evaluate the inflammatory reaction to LPS, we assess glial responses (astrocytes and microglia, including their interaction with the vasculature) in the pons (Fig. 3.7A). There was an acute reactive astrogliosis at LPS1 that extended until LPS3 in the parenchyma and to LPS5 around the microvessels. By LPS7 and LPS9 the $GFAP^+$ staining per unit in both locations was approximately half that of controls (Fig. 3.7B). The closest $GFAP^+$ labeled area fraction to controls was at LPS5, when astrocytes showed long thin processes as seen in Figure 3.7A. Examination of microgliosis revealed that the area occupied by $CX3CR1^+$ cells was markedly increased in association with the microvessels at LPS1 and LPS3 (Fig. 3.7C). A transient decrease was observed in the pons parenchyma at LPS5, when microglia showed a dystrophic morphology (Fig. 3.6A), followed by a remarkable increase at LPS7 and LPS9, but not around the microvessels (Fig. 3.7C). Collectively, these results suggest that the kinetics of astrocytosis and microgliosis are inversely correlated in the parenchyma from LPS1 to LPS9.

### 3.7. Increased density of $CX3CR1^+$ cells around the vessels precedes the loss of $GFAP^+$ cells in pons parenchyma

Our data pointed to a transition from astrocyte gain to loss between LPS5 and LPS7. In order to better understand this transition, we evaluated the glial-vasculature response at an intermediate time point - LPS6 (Fig. 3.8A). At this time point, $GFAP^+$ staining per unit in the parenchyma was similar to control and still higher around the vasculature (Fig. 3.8B) similar to LPS5 (Fig. 3.7B). On the other hand, the parenchymal area occupied by $CX3CR1^+$ cells at LPS6 had already increased to control levels. Surprisingly, the area of microvasculature covered by these cells was significantly increased at this time point (Fig. 3.8C), which was not visible either at LPS5 or at LPS7 (Fig. 3.7C). These data suggest that $CX3CR1^+$ cells migrate towards the microvasculature prior to the loss of perivascular $GFAP^+$ cells. Lastly, we performed ApopTag staining at LPS6 to determine if the significant loss of $GFAP^+$ staining at LPS7 was due to apoptosis. No difference in apoptotic cells was observed between the control and LPS treated mice at this time point, nor did the ApoTag staining co-localize with microglia or astrocytes (Fig. 3.8D,E). Thus, the reduction in $GFAP^+$ staining at LPS7 likely results from a mechanism other than cell death.




C

| | Sholl analysis summary | | | |
|---|---|---|---|---|
| | 1 DAY | | 9 DAYS | |
| | W/O LPS | LPS | W/O LPS | LPS |
| Process Maximum (Nm) | 6.46 ± 0.60 | 7.28 ± 0.52 | 12.59 ± 0.61 § | 10.55 ± 0.41 * |
| Critical Value (µm) | 9.22 ± 1.09 | 8.05 ± 0.33 | 17.39 ± 0.83 § | 12.53 ± 0.53 ** |
| Maximum branch lengh (µm) | 19.17 ± 0.77 | 16.72 ± 0.87 | 26.14 ± 1.63 | 20.13 ± 0.21 ** |
| Number of primary branches (Np) | 4.07 ± 0.45 | 5.30 ± 0.40 | 6.65 ± 0.27 | 5.89 ± 0.19 |
| Schoenen ramification index (Nm/Np) | 1.45 ± 0.13 | 1.44 ± 0.06 | 1.93 ± 0.07 | 1.86 ± 0.05 |

**Figure 3.6 – Morphological changes in microglia following lipopolysaccharide (LPS) administration.** Representative confocal images of CX3CR1$^{+}$ cells morphology from brain cryosections of C57BL/6 CX3CR1$^{gfp/+}$ mice at days 1/3/5/7/9 post-lipopolysaccharide (LPS) administration are shown (A). Using ImageJ software (NIH, USA) we performed Scholl analysis (>40 cells per animal) at days 1 and 9 post-LPS administration (B,C). Results are mean ± SEM from at least four animals. *P < 0.05 and **P < 0.01 *vs*. respective without (W/O) LPS; $^{§}$P < 0.05 and $^{§§}$P < 0.01 *vs*. respective day 1 post-LPS.

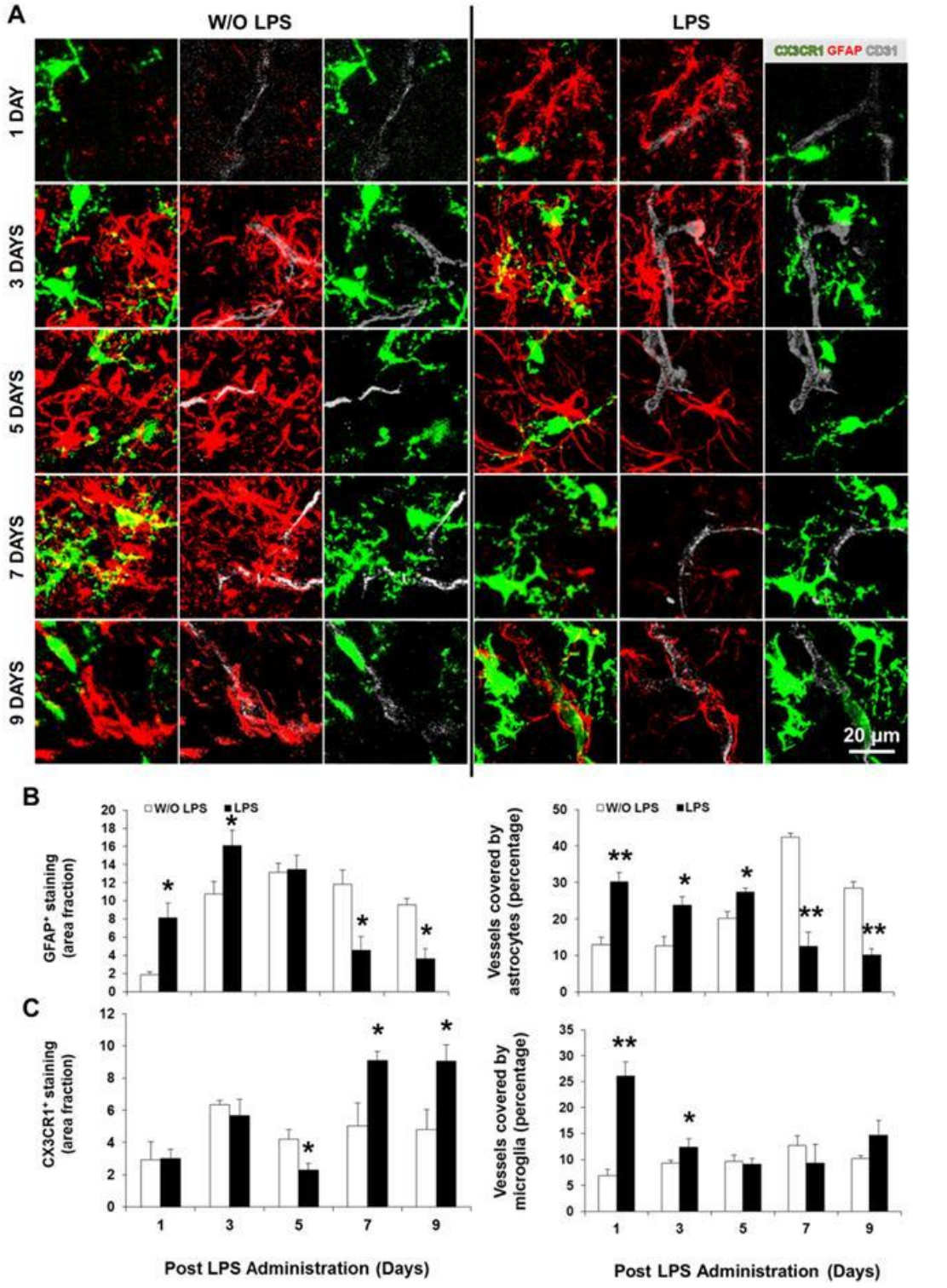



**Figure 3.7 – Early astrogliosis and increased vessel coverage by glia is followed by delayed parenchymal microgliosis and overall astrocytic loss.** Brain cryosections of C57BL/6 CX3CR1$^{gfp/+}$ mice at days 1/3/5/7/9 post-lipopolysaccharide (LPS) administration were immunolabeled for astrocytes (glial fibrillary acidic protein, GFAP, red) along with vessel marker CD31 (cluster of differentiation 31, gray). Representative confocal images of glia-endothelium interactions in pons are displayed in A. Area fraction per field of GFAP and CX3CR1 (green) positive staining, as well as their respective colocalization with the vessels (B,C) were determined by ImageJ software (NIH, USA). Results are mean ± SEM from at least four animals. *P < 0.05 and **P < 0.01 *vs*. without (W/O) LPS.

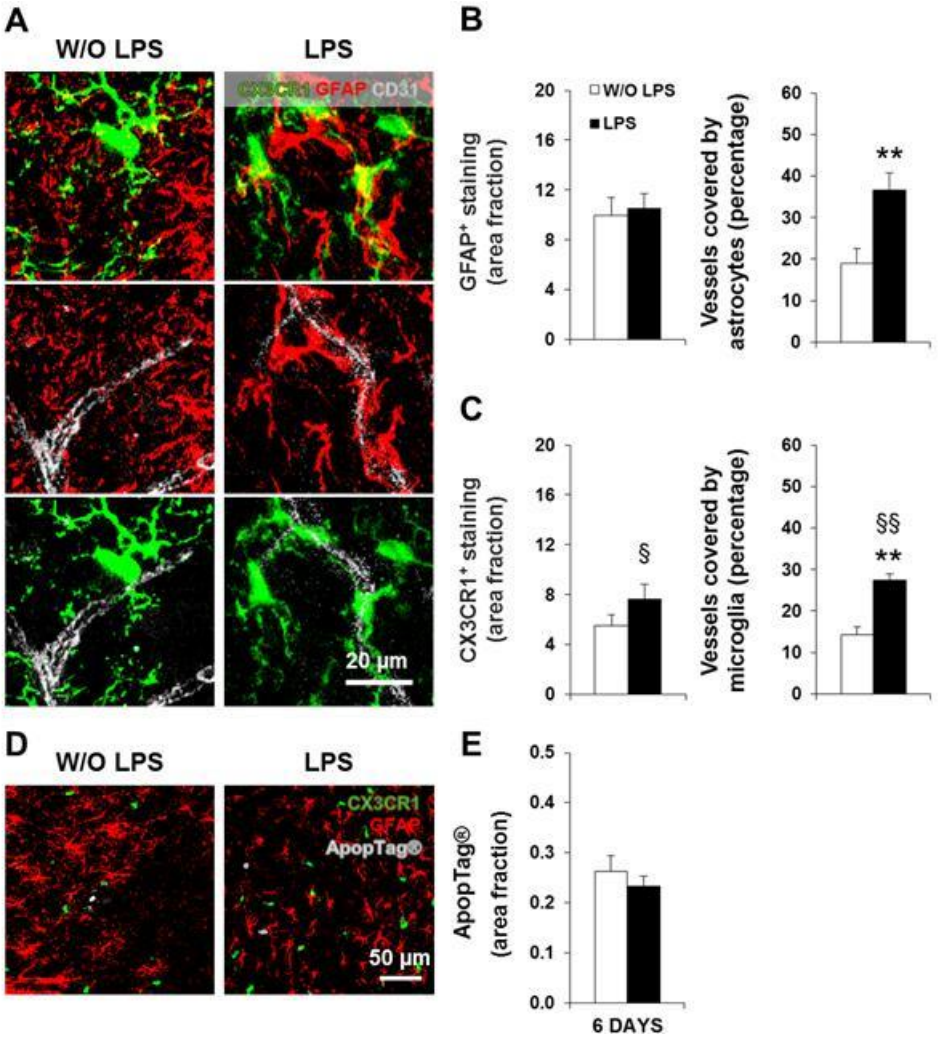



**Figure 3.8 – Loss of astrocytes in neonatal inflammation is preceded by proliferation/migration of CX3CR1$^{+}$ cells in the pons.** Brain cryosections of C57BL/6 CX3CR1$^{gfp/+}$ mice at day 6 post-lipopolysaccharide (LPS) administration were immunolabeled for astrocytes (glial fibrillary acidic protein, GFAP, red) and brain microvascular endothelial cells (cluster of differentiation, CD31, gray). Representative confocal images of each condition in pons are depicted in A. Area fraction per field of GFAP$^{+}$ and CX3CR1$^{+}$ (green) staining, along with their respective colocalization with vessels, were determined using ImageJ software (NIH, USA) (B,C). Parallel sections were immunolabeled for astrocytes (GFAP, red) and for ApopTag (gray) (Representative confocal images in D). Quantification of ApopTag positive staining area fraction, using the above mentioned software, is shown in E. Results are mean ± SEM from at least four animals. **P < 0.01 *vs*. without LPS; $^{§}$P < 0.05 and $^{§§}$P < 0.01 *vs*. day 5 post-LPS.

## 4. Discussion

In the present study, newborn mice were injected with LPS for three consecutive days as an *in vivo* model of sustained neonatal inflammation. Body weight, myelination, neurons, inflammatory biomarkers and glial cells were evaluated for a week after the last injection to establish the impact of peripheral LPS challenge on the developing neonatal brain. The acute loss in body weight indicates that LPS

administration induces a sickness behavior in early neonatal mice. In a study by Du *et al.* (2011), the effects of LPS in newborn mice were also studied, although the exposure period was longer (PND3 to PND11) and less LPS was administered per day (0.3 mg/kg). Nevertheless, the animals examined at PND12 maintained their body weight. The difference in weight observed between our studies is likely due to the lower dose of LPS injected, as other studies have recently reported weight loss in rats injected once at PND5 with 2 mg/kg LPS (Fan et al. 2013).

We also observed an acute loss in brain weight following LPS injection. This is consistent with a study showing that intrauterine administration of LPS (125 µg per dam) in mice at E15 reduces brain weight relative to controls, even at PND14 (Wang et al. 2007). Again, this appears to be a dose-dependent effect, given that intrauterine administration of lower LPS concentrations (80 µg/kg or ~3 µg per dam) does not induce brain weight loss in offspring at PND14 (Graf et al. 2014). The overall loss in brain weight may be linked in part to cerebellar hypoplasia. Several cerebellar regions were reduced in size following LPS administration, and our results suggest that this decrease might result from neuronal atrophy. In addition, we observed that there was a decreased soma area and associated neuronal loss in all brain regions studied. The aforementioned study by Du *et al.* (2011) also demonstrated a loss of $NeuN^+$ neurons. Another study revealed no change in NeuN staining 24 h after exposure to LPS (1 mg/kg) (Chung et al. 2010). It is important to consider, however, the latter study was performed on adult animals, and the negative impact of LPS is likely more pronounced during neuronal development.

We also evaluated how prolonged neonatal inflammation influenced myelination; we observed a lasting effect on MBP levels that extended to LPS9. Most studies to date have evaluated this parameter during the prenatal period (Dilek et al. 2013; Girard et al. 2012; Rousset et al. 2013). Nevertheless, a recent study in mice injected with small doses of LPS (0.05 mg/kg) at PND5, and sacrificed over a week later at PND14, reported a reduction in MBP expression (Pang et al. 2012), which is consistent with our findings. Moreover, another study reported myelin loss in newborn mice injected daily from PND3 to PND11 with 0.3 mg/kg LPS (Du et al. 2011). Based our on data showing no phagocytic uptake of MBP by $CX3CR1^+$ myeloid cells, we believe that LPS does not induce degradation of myelin, but instead slows or arrests the myelination process. Supporting with this theory, we observed an increased number of $NG2^+$ oligodendrocyte progenitors in the cerebellum and pons the day after LPS administration.

To assess the neonatal brain's response to systemic inflammation, we quantified relevant biomarkers and revealed increased expression of MMP-9, TLR4,

and HMGB1 at LPS1. MMPs are gelatinases that have the capacity to remodel the extracellular matrix, promote cellular invasion, and induce various signaling pathways (Sternlicht and Werb 2001). We previously showed *in vitro* that increased release of active MMP-9 and MMP-2 by BMEC occurred within 24 h of LPS exposure (Cardoso et al. 2012). *In vivo* we observed an elevation in MMP-9 but not MMP-2 levels following LPS administration. Previous studies have shown that LPS-stimulated pericytes and microglia can release high levels of active MMP-9 (Milner 2009), which has the potential to disrupt brain homeostasis, degrade the extracellular matrix, and ultimately weaken the BBB, giving rise to leakage (Tilling et al. 1998).

Given that MMP-9 can open the BBB (Rosenberg et al. 1998) and LPS induces its expression through the TLR4/NF-κB pathway (Aid et al. 2010), we examined whether expression of this receptor was elevated in the brain following peripheral LPS challenge. We observed increased expression of TLR4 at LPS1 but not LPS9. TLRs are expressed on immune cells, microglia as well as BMEC and respond to microbial infections. TLR4 is the receptor responsible for the initial inflammatory response to LPS and is usually elevated within hours of exposure (Shimazu et al. 1999). Although few studies have focused on TLR4 expression in newborn mice, a recent study reported in 6-week-old mice that TLR4 was elevated 24 h after exposure to 1 mg/kg of LPS (Chung et al. 2010), which is in agreement with our data.

HMGB1 is another interesting protein because it is an endogenous agonist for TLR4. It is secreted by activated immune cells and endothelial cells as well as stimulated neurons, astrocytes, among others (Andersson and Tracey 2011). HMGB1 was acutely elevated in our brain homogenates at LPS1, which would potentiate TLR4 signaling. Previous studies have demonstrated that this biomarker is markedly increased during neonatal inflammation (Goto et al. 2010; Kato et al. 2009). Interestingly, HMGB1 can induce a loss of body weight (Agnello et al. 2002) and may have contributed to the body weight reduction observed in our study.

In contrast to MMP-9, TLR4, and HMGB1, we observed decreased expression of ATX at LPS1, which contrasts with other studies of inflammatory diseases showing increased ATX expression (Sevastou et al. 2013). A recent study by Awada *et al.* (2012) demonstrated that ATX inhibited microglial activation and induced an anti-inflammatory state. Another study showed that ATX expression inhibited a bacterial endotoxin-induced proinflammatory response (Fan et al. 2008). However, the impact of ATX on inflammatory processes is not entirely understood. In fact, inhibition of ATX is sometimes proposed as a potential therapy for inflammatory diseases (Federico et al. 2008; Gendaszewska-Darmach 2008). Li and Zhang (2009) recently demonstrated that LPS induced ATX expression in a monocytic cell line (THP-1), enhancing cell

migration. Further studies are required to determine the exact role played by ATX in the developing neonatal brain following a peripheral LPS challenge.

Because the inflammatory biomarker profile suggested a role for glial cells in the response to sepsis, we analyzed their reactivity and interactions with brain microvasculature. Both astrocytes and microglia became reactive and increased their coverage of blood vessels early after LPS administration. This response waned over time, with astrocytes eventually showing reduced vascular interactions. A prolonged glial response to LPS was recently described in the hippocampus and brainstem of adult mice following induction of sepsis (Granger et al. 2013). In addition, it has been shown that microglia and astrocytes proliferate in response to LPS (Chung et al. 2010; Nishioku et al. 2009) and *E. coli* (Bland et al. 2010). Our results are in agreement with the findings of Gómez-Nicola *et al.* (2008) who showed that LPS administration alone can induce reactivity of both microglia and astrocytes. It is conceivable that the neuronal atrophy and delayed myelination observed in our study is in fact linked to the LPS-induced glial response. Astrocytes and microglia are essential for the formation, trimming, and function of developing synapses (Christopherson et al. 2005; Miyamoto et al. 2013) as well as for CNS myelination by promoting OPCs migration, proliferation, and differentiation (Pang et al. 2013). Studies have reported that the loss or dysfunction of astrocytes can lead to demyelination or inhibited oligodendrocyte maturation (Lutz et al. 2009; van den Heuij et al. 2014). Others have also shown that disruption of microglia-mediated synaptic pruning contributes to neurodevelopmental disorders (Zhan et al. 2014) and produces long-lasting defects in oligodendrocyte maturation and myelination (Favrais et al. 2011; Volpe 2011).

The divergent early and late astrocytic response to LPS administration was particularly interesting given the role that these cells play in brain homeostasis and the maintenance of the BBB. Sherwin *et al.* (2005) showed that LPS binds to microglia and astrocytes during neonatal neuroinflammation, and for astrocytes this is particularly intense around blood vessels. Importantly, astrocytes can reduce BBB permeability following LPS administration (Kaya et al. 2004). Therefore, we postulate that the initial increased astrocytic coverage of microvessels observed in our study could be a protective reaction to neuroinflammation. Surprisingly, this response declined over time, which coincided with increased $CX3CR1^+$ staining and distribution. This change in microglia could be due to the release of chemoattractants by astrocytes. Astrocytes express CX3CL1, the ligand for CX3CR1 on microglia/macrophages. In response to proinflammatory cytokines, CX3CL1 expression is upregulated in cultured astrocytes and induces microglia chemotaxis (Hulshof et al. 2003). It is also important to note that the reduced association of $GFAP^+$ cells with microvessels coincided with the

morphological transformation of microglia into a bushy activated state. Microglial activation was previously described as a modulator of astrocytic proliferation, in the same manner as astrocytes can modulate microglial functions such as cell proliferation, migration, and cell adhesion [see for review: (Seifert et al. 2006)]. In fact, it has been shown that 7 days following a neuroinflammatory stimulus, adult animals with reduced astrogliosis show increased microglial activation and neuronal loss compared to controls (Lu et al. 2011; Shi et al. 2012). Our failure to detect astrocyte apoptosis in the pons at the time of reduced $GFAP^+$ staining suggests astrocytic deactivation or dedifferentiation. There is evidence that certain stimuli released by injured astrocytes can reprogram them into neural stem cells, which can then be redifferentiated into neuronal and glial cells, even in adult animals (Corti et al. 2012; Heinrich et al. 2010; Seri et al. 2001). This could explain the rapid increase in the number of neurons and microglia in our model.

## 5. Conclusions

In summary, our data demonstrate that systemic inflammation profoundly alters several anatomical and inflammatory aspects of the brain when experienced during the early neonatal period. Our results not only expand upon previous literature focused on the acute effects of LPS on the developing brain, but are also the first to document the progressive neuroinflammatory changes that occur in the week that follows LPS administration. The reciprocal relationship between astrocytes and microglia is interesting and warrants follow up studies to identify the nature of the cross-talk. It will also be important to determine the degree to which the observed changes contribute to long term sequelae following neonatal sepsis and whether these changes can be reversed therapeutically in afflicted patients.

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*Chapter 4*

# TIME-POINT OF NEONATAL SYSTEMIC INFLAMMATION IS A DETERMINANT OF THE NEURODEVELOPMENTAL OUTCOME AND GLIAL RESPONSE IN YOUNG ADULT MICE

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## Abstract

Systemic inflammation during the early neonatal period (first week of life) induces acute neuronal degeneration and atrophy, in addition to altering glial response. However, the lasting effects resulting from impairment of mouse neurodevelopment after having this condition in either the first or the second week of life, remain unknown. Therefore, we aimed to determine the acute effects of lipopolysaccharide (LPS)-induced inflammation during the second of life [examined at postnatal day (PND)14], as well as its lasting effects in young adults (PND53) (with onset during the first or the second week of life). Also, we intended to study the primed response to a second pro-inflammatory stimulus at the same adult age compared to an adult acute response (animals were injected at PND52 and examined at PND53). Newborns had neuronal loss and atrophy as well as white matter damage. In addition, LPS triggered an acute pro-inflammatory response with associated astrogliosis and microgliosis. Regarding the lasting effects of this clinical condition, animals injected at the first week had mainly neurodegeneration, whereas those injected in the second week of life also depicted lasting injury to the white matter and loss of astrocytes. At last, we evaluated the primed response to a second inflammatory challenge. Animals primed in the first week had little effects compared to a single adult injection. On the other hand, animals primed during the second week of life not only had damage to the white matter, but also neuronal atrophy and deficient glial response. Thus, the window of neonatal systemic inflammation determines the lasting and primed effects in the different regions of the CNS.

## 1. Introduction

During the perinatal period, the developing brain is particularly vulnerable to inflammatory conditions. In fact, approximately 25-40% of preterm births (before 37 weeks of gestation in humans) are associated with a maternal immune response to an infection (Goldenberg et al. 2008; Nimmervoll et al. 2013). In general, the sequence of neurodevelopmental events is comparable among species (Rice and Barone 2000). At birth, the neurological maturity of rodent pups is similar to that of human preterm neonates at 24 weeks of gestation (Anand et al. 1999). The first week of rodent life (early neonatal period, ENP) corresponds to the third gestational trimester of human fetus, whereas the $2^{nd}$-$4^{th}$ rodent weeks (late neonatal period, LNP) can be compared to a term human newborn (Pressler and Auvin 2013; Rodekamp et al. 2005). During this critical neurodevelopmental window, one of the most common clinical conditions afflicting newborns is sepsis (Black et al. 2010).

Sepsis is a severe systemic inflammatory response in attempt to eliminate pathogens (Stevenson et al. 2014). Sepsis-associated encephalopathy (SAE) manifests with a rapid decline in cognitive functions and may lead to permanent neurocognitive dysfunction and functional impairments, even after the patient has recovered (Widmann and Heneka 2014). Inflammatory mediators disrupt the blood–brain barrier (BBB) during the early stages of SAE. Thus, several innate immune components can enter the brain not only through specific receptors but also due to the disruption of the endothelium itself (Cardoso et al. 2010; Fox et al. 2013; Nayak et al. 2012; Nishioku et al. 2009), further inducing neuronal and glial dysfunction (Semmler et al. 2008; Stocchetti 2005). Reducing inflammation during sepsis improves brain functional recovery, hinting that SAE may indeed be a consequence of the inflammatory response (Boos et al. 2005; Deng et al. 2013).

Regarding the effects of SAE in the premature brain, surviving infants are at higher risk for long-term neurodevelopmental disability due to the lack of antioxidant defense mechanisms, the poorly developed germinal matrix, as well as immature oligodendrocytes and astrocytes (Alshaikh et al. 2013; Leviton and Dammann 2004). Microglia are also maturing at this stage, with its transition from an amoeboid into a ramified shape only occurring during the second week of rodent life (Harry and Kraft 2012; Nayak et al. 2014; Neiva et al. 2014). Still, during the first week of life, newborns profit from maternal factors that aid in the immune defense (Battersby and Gibbons 2013). On the other hand, neonates who develop sepsis during LNP have higher mortality rates, especially if infected with gram-negative organisms (up to 40%) (Stoll et al. 1996). During these later stages, the development of the central nervous system

(CNS) focuses on neuronal activity (synaptogenesis and myelination) and on glial proliferation and differentiation (Rice and Barone 2000).

*In vivo* experiments have demonstrated increased BBB permeability alongside neuronal damage induced by inflammation in the developing brain (Bilbo et al. 2008; Stolp et al. 2005a; Stolp et al. 2005b). Our own previous (and still unpublished) studies revealed a late pro-inflammatory response with strong glial interference following an ENP systemic inflammation. Thus, disturbances during this neurodevelopmental window may lead to severe damages that can be irreversible (Kroon et al. 2013; Rice and Barone 2000).

To better understand the lasting cerebral consequences of neonatal SAE, in this study we aimed to determine its influence on the young adult neuronal and glial development, and whether such response depend on an initial ENP or LNP inflammation. Simultaneously, we evaluated the acute response of primed animals to a second stimulus, also varying the window of priming.

## 2. Materials and Methods

### 2.1. Animals

CD1 mice were purchased from Harlan Ibérica laboratories (Spain). Mice were housed with a 12 hour light/dark cycle and were provided with *ad libidium* access to a standard laboratory chow diet and drinking water. This study was performed in accordance with the European Community guidelines (Directives 86/609/ECC, European Convention for the Protection of Vertebrate Animals used for Experimental or Other Scientific Purposes) and Portuguese Laws on Animal Care (Directive 1005/92). All experimental procedures were performed under anesthesia, conducted in a manner to minimize animal suffering and all efforts were made to use the minimum number of animals.

### 2.2. Drug Administration

The day of birth of the mice was defined as postnatal day (PND)1. Offspring of both genders were randomly divided and received intraperitoneal injections of either endotoxin-free saline (SF) or of lipopolysaccharide (LPS) [6 mg/kg, Escherichia coli 055:B5; Calbiochem (Merck, Germany)] to induce systemic inflammation (Erickson et al. 2012; Jin et al. 2011). We followed three studies with different injection plans, as seen in **Figure 4.1**, with $n \geq 4$ animals in each condition per analysis.

Given our previous (still unpublished) data on the acute effects on neurodevelopment during a system inflammation in the first week of life (ENP), we

initially aimed to determine the acute effects of neuroinflammation during the second week of life (LNP). Mice were injected with LPS daily at PND11,12,13, and were examined the following day (PND14). We also intended to evaluate whether moving the window of exposure to LPS from the first to the second week of life would trigger different lasting impairments on neurodevelopment. Animals were injected with LPS during the first week (at PND4,5,6) or during the second week (PND11,12,13) and were examined at young adult age, more precisely at PND52. Lastly, we aimed to determine if a primed response during the neonatal period would differ from an acute adult response, and whether the induced effects depended on the neonatal period of the priming. Newborn animals were separated into two groups, and each received either the first- or the second-week LPS-administration plan. At PND52, the same animals received a new LPS challenge and were examined the following day (PND53).

All animals were weighed before perfusion for posterior comparison of body weight. Whole brains were also weighed following perfusions.

### 2.3. Tissue Process

For gelatin zymography and western blot analysis, animals were anesthetized with a lethal dose of Pentobarbital and intracardially perfused with phosphate buffer saline solution, pH 7.4 (PBS). Brains were quickly removed and snap-frozen followed by cryopreservation at -80ºC for at least 24 h. Protein extracts were obtained by lysing the brain tissue with radioimmuno-precipitation assay (RIPA) buffer (Tris Buffer 1 M pH 8.0, EDTA 0.5 M pH 8.0, NaCl 5 M, 10% NP-40, 50% glycerol, 10% SDS). For histological analysis, the perfusion was performed with 4% paraformaldehyde (PFA) in PBS. Brains were post-fixed in the indicated fixative for at least 24 h. Brain tissue was processed for paraffin and cut into 6 µm sagittal sections.

### 2.4. Staining for Luxol Fast Blue and Cresyl Violet

Paraffin sections were stained with Luxol Fast Blue (VWR, USA) and with Cresyl Violet (Sigma, USA) for determination of oligodendrocyte myelination in white matter and neuronal Nissl bodies'. Sections were rehydrated before overnight incubation with 0.1% Luxol Fast Blue solution, at 56ºC. After washing, slides were differentiated in a 0.5% lithium carbonate solution, followed by 2% acetic acid solution for 5 min. Next, slides were counterstained with a 1% Cresyl Violet solution for 10 min, followed by differentiation in 37% acetic acid and a final wash in distilled water. Tissue sections were mounted with Fluoromount-G (Southern Biotech, USA) and visualized using a DFC 490 camera (Leica, Germany) adapted to an Axioskop bright field microscope (Zeiss, Germany). Using ImageJ 1.48x software (NIH, USA). Width of each cerebellar

layer was evaluated in photos with magnification of 100x. The numbers of neurons per field and their soma area in the cerebellar Purkinje layer, CA3 hippocampal region and throughout the pons regions were quantified in photos of 400x magnification.

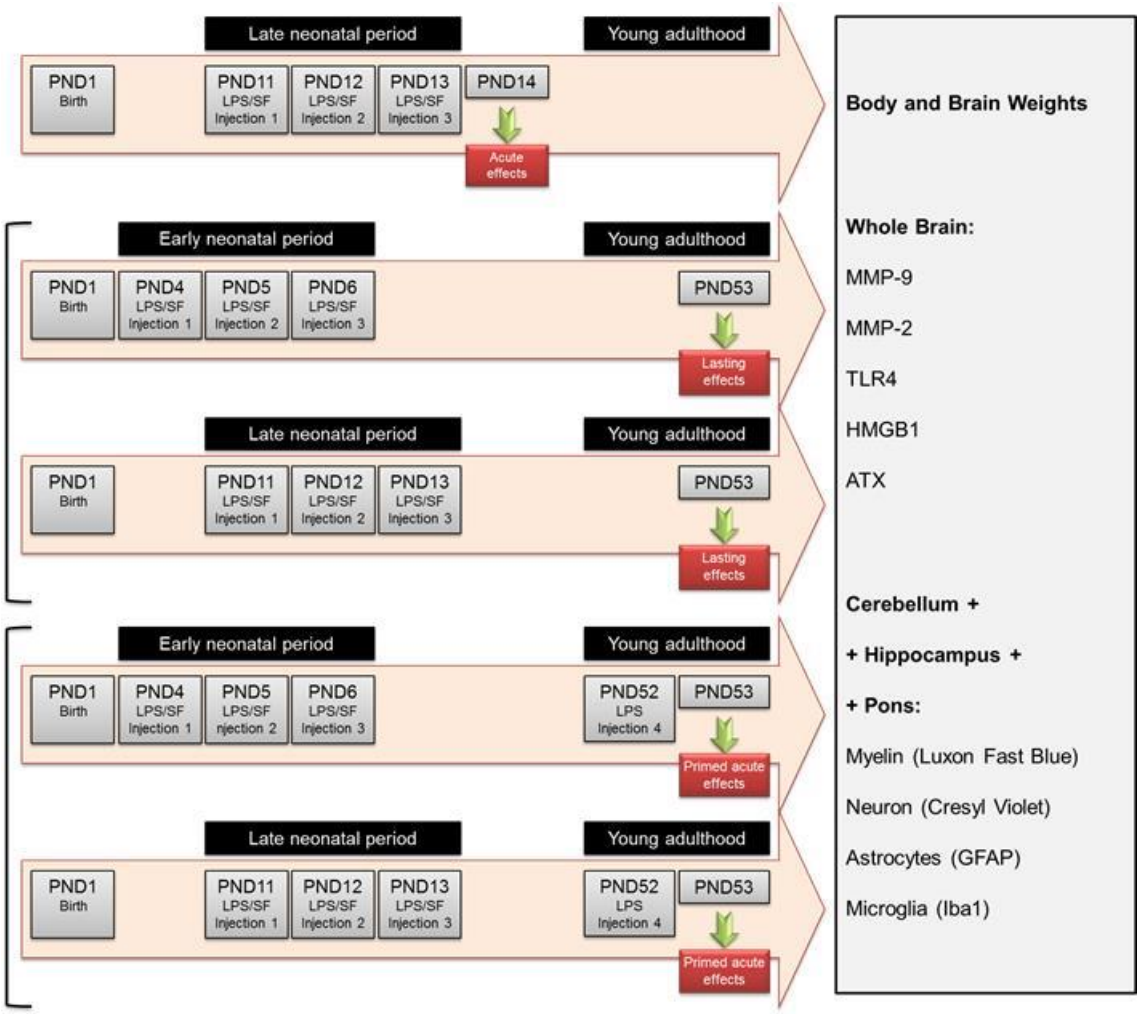


**Figure 4.1 – Schematic representation of lipopolysaccharide (LPS) administration plans and evaluated parameters.** Mice were given endotoxin-free saline (SF)/LPS injections according to different injection plans: at the postnatal days (PND) 4, 5 and 6, or at PND 11, 12 and 13, corresponding to early or late neonatal periods, respectively. Mice were examined the day after the last neonatal injection (PND14) to study acute effects, or at PND53 to determine the lasting consequences. A second LPS injection was administered at PND52 and mice were examined the day after (PND53) to evaluate the primed effects. Body weight, activity and expression of inflammatory biomarkers in whole brain [matrix metalloproteinase(MMP)-9 and -2, toll-like receptor (TLR)4, high mobility group box 1 (HMGB1) and autotaxin (ATX)], as well as alterations in white matter, neurons, astrocytes and microglia were evaluated in the cerebellum, the hippocampus and the pons at each time point.

### 2.5. Gelatin Zymography

For evaluation of the activity of matrix metalloproteinase (MMP)-9 and MMP-2, 40 µg of protein from tissue extracts were analyzed by SDS-PAGE zymography in 0.1% gelatin/10% acrylamide gels under non-reducing conditions. After electrophoresis, gels were washed for 1 h with 2.5% Triton X-100 (in 50 mM Tris pH 7.4, 5 mM $CaCl_2$, 1 mM $ZnCl_2$) to remove SDS and renature the MMPs species in the gel. Gels were next incubated in developing buffer (50 mM Tris pH 7.4, 5 mM $CaCl_2$, 1 mM $ZnCl_2$) for 72 h at 37ºC to induce gelatin lysis. For enzyme activity analysis, gels were stained with 0.5% Coomassie Brilliant Blue R-250 (Bio-Rad, USA) for 3 h at room temperature (RT), and distained in 30% ethanol/10% acetic acid solution. Gelatinase activity, detected as a white band on a blue background, was quantified by computerized image analysis using Quantity One 1-D Analysis Software (Bio-Rad, USA).

### 2.6. Western Blot

Western blot analysis was performed using 100 µg of protein from tissue extracts. Proteins were separated on a 12% SDS-PAGE gel and following electrophoretic transfer onto a nitrocellulose membrane and blocking with 5% milk solution, the blots were incubated with primary antibody overnight at 4ºC [rabbit anti-Toll-like receptor(TLR)-4 (Santa Cruz, USA, #sc-10741; 1:500), mouse anti-high mobility group box (HMGB)-1 (BioLegend, USA, #651402; 1:500), rabbit anti-autotaxin (ATX) (Millipore, USA, #ABT28; 1:500) or mouse anti-β-actin (Sigma, USA, #A5441; 1:5000)] and with horseradish peroxidase-labelled secondary antibody [anti-mouse or anti-rabbit (Santa Cruz, USA, #sc-2005 and #sc-2004 respectively; 1:5000] for 1 h at RT. Protein bands were detected by LumiGLO® (Cell Signaling, USA) and visualized by chemiluminescence with ChemiDoc (Bio-Rad, USA). Expression was quantified by computerized image analysis using Quantity One 1-D Analysis Software (Bio-Rad, USA).

### 2.7. Paraffin-Immunofluorescence

Paraffin sections were used to assess the expression of GFAP for astrocyte detection and of ionized calcium binding adaptor molecule (Iba)1 for microglia staining). Briefly, heat antigen retrieval was performed in rehydrated tissue sections (3.7 mM sodium citrate, 1 mM EDTA, pH 6.0). Tissue was incubated overnight at 4ºC in 1% bovine serum albumin (BSA) (Sigma, USA) + 0.1% Triton-X100 (Roche) with primary antibodies mouse anti-GFAP (Novocastra, Leica, Germany, #GFAP-GA5, 1:100) and rabbit anti-Iba1 (WAKO, Japan, #019-19741; 1:250). Sections were incubated with secondary antibody Alexa 488 anti-rabbit IgG or Alexa 594 anti-mouse IgG (Invitrogen,

USA, #A11008 and #A11005 respectively; 1:500), overnight at 4ºC. Nuclei were counterstained with DAPI dye. After dehydration, slides were mounted with DPX (BDH Prolabo, UK). Images were captured using an AxioCam HRM camera (Zeiss, Germany) adapted to an AxioScope.A1 microscope (Zeiss, Germany). Images were collected using sequential scanning with the 405, 488 and 594 nm laser lines to produce three color overlays. Cell counting was performed using a magnification of 630x. Immunopositive cells with identified nuclei counterstaining by DAPI were counted throughout the cerebellum, the hippocampus and the pons regions using Image J 1.48x Software (NIH, USA). Results are presented as number of cells per field.

### 2.8. Statistical Analysis

Results are expressed as means ± SEM from, at least, four independent animals in each LPS-treatment group. Significant differences between control and LPS-treated animals for each time point were determined by the two-tailed *t*-test performed on the basis of equal and unequal variance as appropriate. Comparison of more than two groups was done by ANOVA one-way with Bonferroni post-hoc test using GraphPad Prism® 5.0 (GraphPad Software, San Diego, CA, USA). Statistical significance was considered when P values were lower than 0.05.

## 3. Results

### 3.1. LPS administration during the second week of life acutely alters the normal morphology of the CNS

The second week of life in the mouse species comprises the beginning of glial maturation, namely the ramification of microglia (Harry and Kraft 2012) and the trimming of overlapping astrocytic processes (Bushong et al. 2004). To better understand if the changes seen in our previous study (Chapter 3) depend on the neonatal window of LPS-treatment, we here evaluated the acute response to systemic inflammation induced during the second week of life. We first determined body and brain weights but did not observe any changes.

*Exposure to LPS in the second week of life leads to acute neurodegeneration and damage of the white matter.* Given that LPS induces acute neurodegeneration when administrated at the first week of life (Chapter 3), we also assayed the number of neurons and their soma area following treatment with the endotoxin in the second-week. As depicted in **Figure 4.2,** PND14 animals had significant neuronal loss in the pons and the cerebellum (0.9-fold, $P<0.05$) (**Fig. 4.2B**), as well as an acute atrophy in

the soma area of neurons from the pons (**Fig. 4.2C**). Since our previous data also indicated that a first-week exposure to LPS delays myelination, along with the shrinkage of neuron-containing cerebellar layers (Chapter 3), we next analyzed cerebellar changes in animals exposed to LPS on the second-week. Curiously, PND14 mice had an acute shrinkage of the white matter layer (~0.5-fold) in addition to lighter staining of Luxol Fast Blue that suggests myelin loss (**Fig. 4.2D).** No further acute alterations were observed in the remaining cerebellar layers (data not shown). These results point to a greater neuronal susceptibility in the pons of animals treated in the second week of life, whereas the cerebellar region hints a particular vulnerability in the white matter.

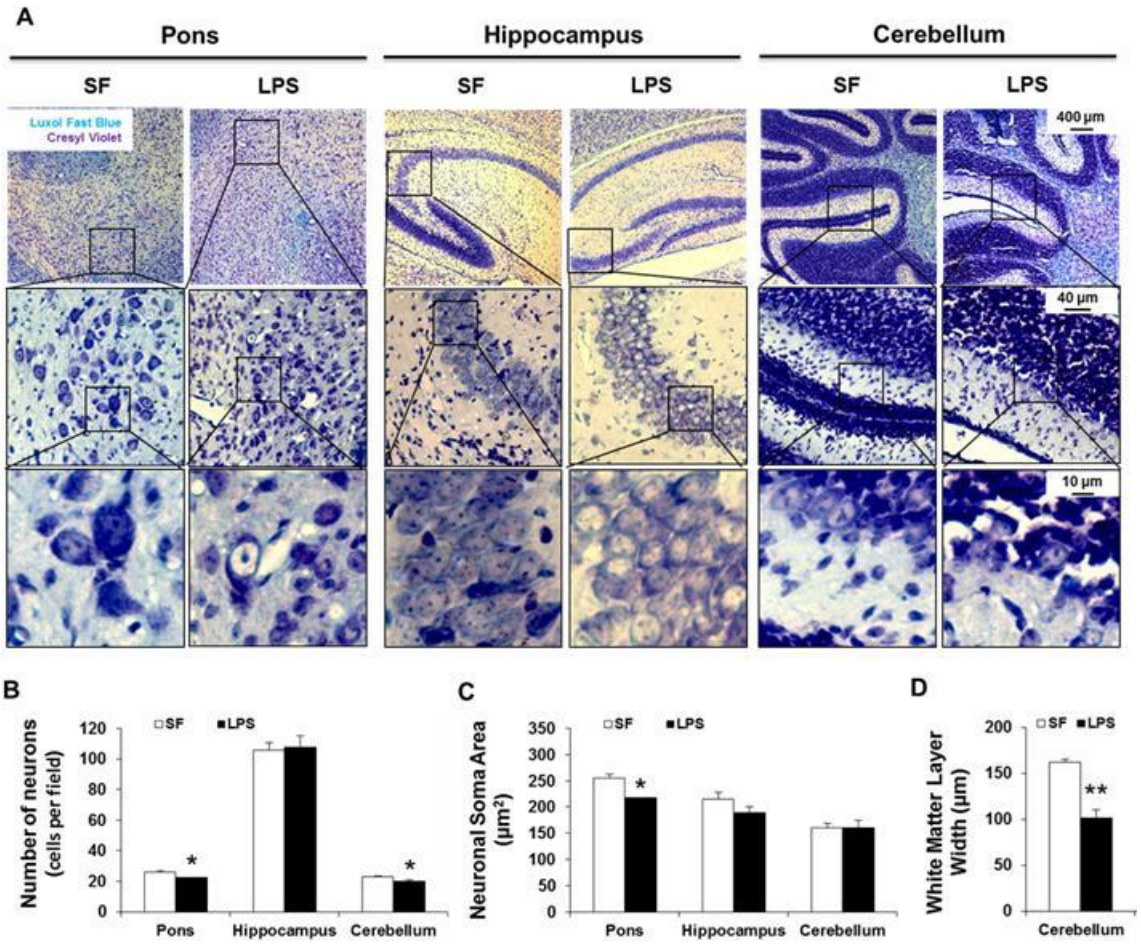


**Figure 4.2 - Late neonatal systemic inflammation triggers acute neurodegeneration and demyelination in newborn animals.** Paraffin sections from newborn mice that exposed to lipopolysaccharide (LPS)-administration in the late neonatal period (PND14), were stained with Luxol Fast Blue (myelin) followed by Cresyl Violet (Nissl bodies'). Representative images of the pons, the hippocampus and the cerebellum are shown in A. Number of neurons per field and their soma area were evaluated (B,C). Width of the white matter layer of the cerebellum was measured and results expressed in µm (D). All results are mean ± SEM of at least four independent experiments. *P <0.05 and **P <0.01 *vs*. respective control (endotoxin-free saline, SF).

*LPS-induced systemic inflammation in the second week of life triggers a mild pro-inflammatory response.* As seen in our previous study (Chapter 3), neuroinflammation during the first week of life enhances the activity or expression of the inflammatory biomarkers MMP-9, TLR4 and its mediator HMGB1. On the other hand the expression of ATX was decreased in response to the neonatal inflammation. Thus, we further evaluated the reflex of a system inflammation induced during the second week of life in these inflammatory biomarkers (**Fig.4.3**). At PND14 MMP-9 was slightly though significantly increased (1.1-fold, $P<0.05$) whereas MMP-2 remained at control levels. Surprisingly, TLR4 was unaltered in spite of the significant increased expression of HMGB1. As for ATX, the decrease in its expression was not statistically significant. Thus, there appears to be a different acute inflammatory response between the first and the second week of life.

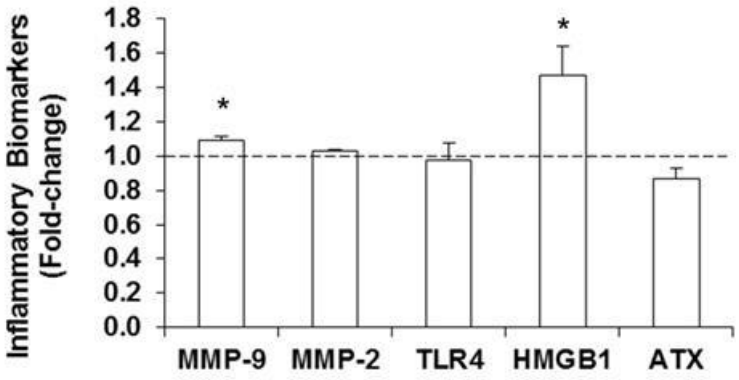


**Figure 4.3 - Systemic inflammation in the second week of life triggers a mild pro-inflammatory response with increased activity of matrix metalloproteinase (MMP)-9 and overexpression of high-mobility group box (HMGB)-1.** Whole brain lysates of newborn mice with lipopolysaccharide (LPS)-administration in the late neonatal period (PND14) were used to determine the activity of MMP-9 and MMP-2 by gelatin zymography, alongside with the expression of inflammatory biomarkers Toll-like receptor (TLR)4, HMGB1 and autotaxin (ATX) by western blot. Results are expressed as fold-change from control ± SEM from at least four animals. *P <0.05 *vs*. control.

*An LPS-challenge during the second week of life promotes the proliferation of glial cells.* MMP-9 was revealed to contribute to the inflammatory response by glia activation (Annese et al. 2014). Thus, and despite the fact that TLR4 and ATX were unaltered at the times studied, we evaluated the acute response of microglia and astrocytes to an LPS-challenge during the second week of life, based on immunofluorescence of $GFAP^+$ astrocytes and $Iba1^+$ microglia in the newborn mouse brain (**Figure 4.4)**. There was an acute astrogliosis at PND14, mainly in the pons (~2-fold) followed closely by the hippocampus and the cerebellum. Regarding microglial response, both the pons

and the hippocampus depicted an acute increase compared to SF treated newborns (~1.5-fold). These results point to a possible connection between exacerbated glial response in the pons and the observed neurodegeneration in this region.

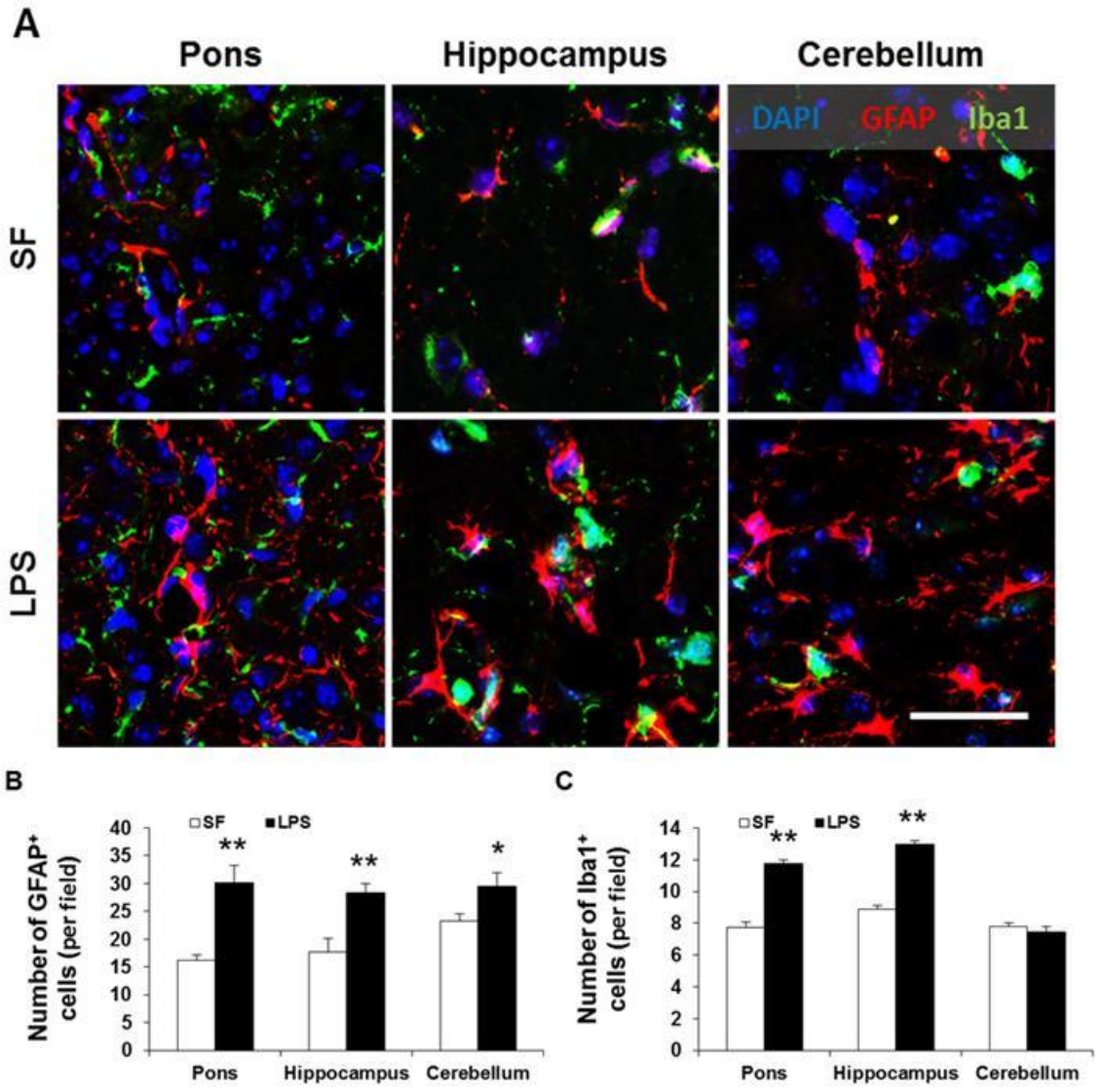


**Figure 4.4 - Late neonatal systemic inflammation induces acute astrogliosis and microglia proliferation/migration.** Paraffin sections from newborn mice that exposed to lipopolysaccharide (LPS)-administration in the late neonatal period (PND14) were immunolabelled for astrocytes (glial fibrillary acidic protein, GFAP, red) and for microglia-like cells (ionized calcium binding adaptor molecule, Iba1, green). Nuclei were counterstained with DAPI (blue). Representative images of the pons, the hippocampus and the cerebellum are shown in A. Number of $GFAP^+$ cells and of $Iba1^+$ cells in the parenchyma, per field, were determined (B,C). All results are mean ± SEM of at least four independent experiments. *P <0.05 and **P <0.01 *vs*. respective control (endotoxin-free saline, SF).

### 3.2. LPS challenge in the first or second weeks of life originate different lasting effects.

Injury to the developing CNS is frequently associated with long-term neurological deficits (Nimmervoll et al. 2013; Volpe 2003; Zavitsanou et al. 2013). In order to assess the impact of LPS exposure in the first or second weeks of life, we evaluated the lasting neuronal and white matter effects, as well as the density of glial cells, in young adult mice (PND53).

*LPS administration in the first week of life induces lasting neurodegeneration in the brains of young adult mice.* Our data revealed that a LPS-challenge in the first week of life induced lasting neuronal loss in hippocampal CA3 region and cerebellar Purkinje layer (**Fig. 4.5A**). Though the pons did not present neuronal loss, there was lasting neuronal soma reduction (**Fig. 4.5B**). We also searched changes in the width of cerebellar layers, in body and brain weights, in the activity and expression of inflammatory biomarkers, as well as in the number of $GFAP^+$ cells and $Iba1^+$ cells. Still, no other lasting effects were found (data not shown).

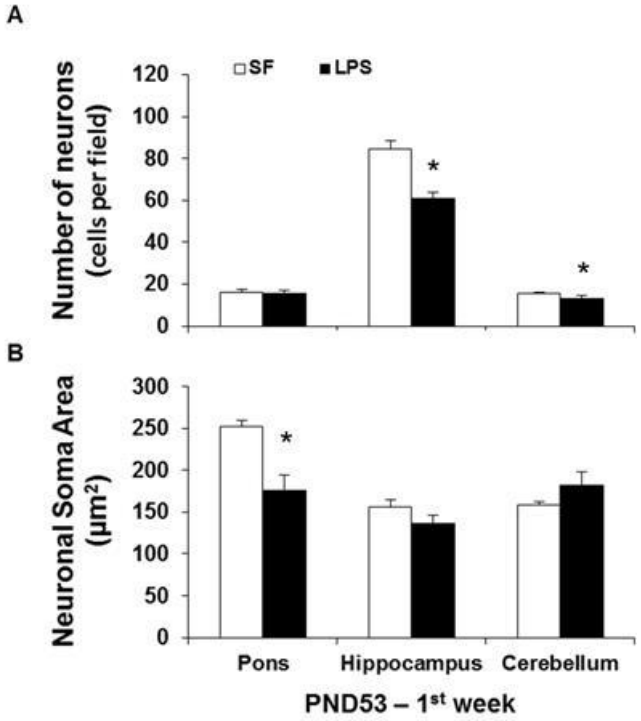


**Figure 4.5 - Systemic inflammation in the first week after birth induces lasting neuronal loss in the hippocampus and the cerebellum, in addition to soma atrophy in pons.** Paraffin sections from young adult mice (PND53) with lipopolysaccharide (LPS)-administration in the first week of life, were stained for neurons with Cresyl Violet (Nissl bodies). Number of neurons per field in the pons, CA3 region (hippocampus) and Purkinje layer (cerebellum) were determined (A), as well as their soma area (B). All results are mean ± SEM of at least four independent experiments. *P <0.05 *vs*. respective control (endotoxin-free saline, SF).

*LPS-induced inflammation in the second week of life leads to lasting neuronal atrophy, shrinkage of the cerebellar white matter layer and loss of astrocytes.* Given that young adult mice exposed to systemic inflammation in the first week of life had lasting neurodegeneration, we analyzed young adult mice subjected to LPS-challenge during the second week. Alike young adult animals with LPS-treatment in the first week, changing the window of injections to the second week does not induce last effects on body and brain weights, nor on the activity and expression of inflammatory biomarkers. As for the effects on neurons, our data revealed that the neuronal atrophy seen in the pons of PND14 animals is still significant at young adulthood (**Fig. 4.6A**). We further analyzed the cerebellar consequences, more precisely on the width of each layer, and observed a lasting shrinkage of the white matter layer at PND53 (**Fig. 4.6B)**, with no further alterations in the remaining layers (data not shown). These results point to different neuronal lasting effects depending on the neonatal week at which exposure to LPS occurs, with the first week being more demising. On the other hand there appears to be a bigger susceptibility of the white matter when subjected to an inflammatory stimulus in the second week of life.

Surprisingly, these young adult animals also had persistent reduced numbers of $GFAP^+$ astrocytes in the hippocampus and the cerebellum, but no lasting effects were found regarding the number of $Iba1^+$ cells (**Fig. 4.6C**). These results, along with the lasting effects observed in the animals that were exposed to LPS in the first week of life, may unveil a possible connection between the degree of white matter demise and the reactivity of astrocytes.

### 3.3. Animals treated with LPS during the second week of life are more susceptible to a second LPS-challenge.

The consequences of priming the immune system are still not consensual. Though some defend that the priming of inflammatory response by LPS may exert a protective effect (Ahmed et al. 2000), others advocate that it results in an exaggerated local inflammatory response (Puntener et al. 2012). This led to us to evaluate the response of young adult animals primed during the first or second week of life to a second LPS-challenge at PND52, compared to an acute adult response at the same age (SF during the neonatal period and only one injection of LPS at PND52).

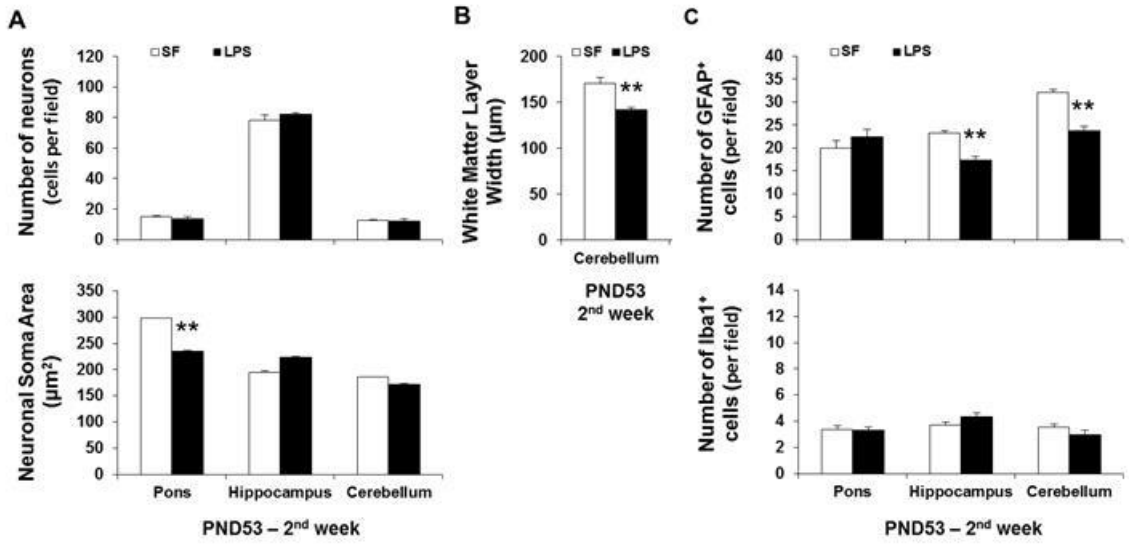


**Figure 4.6 - Late neonatal systemic inflammation triggers lasting neuronal atrophy in the pons, cerebellar demyelination, in addition to decreased numbers of astrocytes.** Paraffin sections from young adult mice (PND53) with lipopolysaccharide (LPS)-administration in the late neonatal period , were stained with Luxol Fast Blue (myelin) and Cresyl Violet (Nissl bodies), to determine the number of neurons per field and their soma area (A). Width of the white matter layer of the cerebellum was also measured and results are presented in µm (B). In parallel, other paraffin sections were immunolabelled for astrocytes (glial fibrillary acidic protein, GFAP, red) and for microglia-like cells (ionized calcium binding adaptor molecule, Iba1, green). Nuclei were counterstained with DAPI (blue). Number of $GFAP^+$ cells and of $Iba1^+$ cells in the parenchyma were determined per field (C). All results are mean ± SEM of at least four independent experiments. *P <0.05 and **P <0.01 *vs*. respective control (endotoxin-free saline, SF).

*Second LPS-challenge induces loss in body weight.* We first studied the physical response by determining body and brain weights, with no changes in the latter. We observed that a second LPS-challenge at PND52 induced additional loss in body weight in animals that were primed during the first week of life compared to an adult acute response (**Table 4.1**). Even though animals primed at the second week of life displayed a similar body weight to those primed in the first week, the correspondent adult acute LPS-treatment also induced loss of body weight [0.9-fold compared to no LPS administration (SF at neonatal period and SF at PND52), $P < 0.01$].

*Priming in the first week of life sensitizes cerebellar neurons, whereas priming in the second week reduces the cerebellar white matter layer.* Next, we evaluated the outcome of a double LPS-challenge on neurons and white matter. Concerning the effects on neurodevelopment, we found no differences in the number of neurons in primed animals compared to an adult acute response, apart from an increase in Purkinje neurons in animals that were primed in first week of life (**Fig.**

**4.7A**).Interestingly, we observed an exacerbated atrophy of the neuronal soma in these same Purkinje neurons (**Fig. 4.7B**). On the other hand, animals that were primed during the second week only presented neuronal atrophy in the hippocampus. Thus, neurons appear to be vulnerable both in animals primed during the first and during second weeks, though with differences between brain regions.

Afterwards, we measured the width of each cerebellar layer (**Fig. 4.7C**). Interestingly, we observed different outcomes depending on the age of priming. After a second LPS-challenge, animals primed during the first week had significantly narrower cerebellar layers, more specifically the molecular, Purkinje neuronal and internal granular layers. On the other hand, animals that were primed during the second week of life had narrower internal granular and white matter layers after a second LPS-exposure. As for the previous parameter, the neurons in the cerebellum are more susceptible to inflammation when primed in the first week.

**Table 4.1 - Priming of the inflammatory response during the first week of life triggers weight loss following a second challenge.**

| Body weight (g) | | |
|---|---|---|
| | Acute adult | Primed adult |
| 1st week | 29.9 ± 5.6 | 23.9 ± 2.7** |
| 2nd week | 25.2 ± 2.3 | 25.5 ± 1.9 |

Animals were weighed (g) after lipopolysaccharide-challenge. All results are the mean value ± SEM of at least eight independent experiments. ***P* <0.01 *vs.* respective adult acute response.

*Second LPS-challenge leads to enhanced pro-inflammatory response in animals primed during the second week of life.* We further determined the activity or expression of the abovementioned inflammatory biomarkers (**Fig. 4.8A**), we observed that animals primed during the first neonatal week had little pro-inflammatory response to the second stimulus, but presented a robust increased expression of ATX when compared to an adult acute response. Animals primed during the second week also had high levels of ATX, as well as of HMGB1.

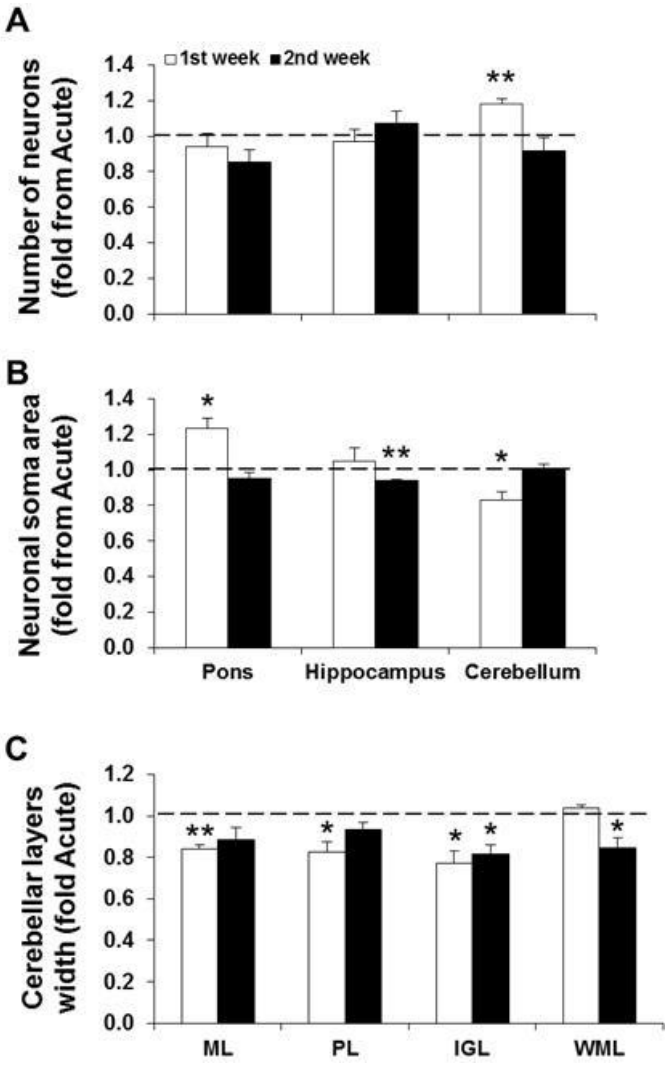


**Figure 4.7 - The type of effects on neurons and the regions affected by a primed response on young adult mice depend on the neonatal week of priming.** Paraffin sections from young adult mice that with lipopolysaccharide (LPS)-administration in the first or second weeks of life followed by a second LPS-challenge at adult age, were stained with Luxol Fast Blue (myelin) followed by Cresyl Violet (Nissl bodies). Number of neurons per field and their soma area were evaluated (A,B). Width of the cerebellar molecular (ML), Purkinje (PL), internal granular (IGL) and white matter (WML) layers was measured in µm (D). All results are fold-change from respective adult acute response ± SEM of at least four independent experiments. *P <0.05 and **P <0.01 *vs*. adult acute response.

*Second LPS-challenge induces different astrocyte and microglia responses depending on the age of priming.* Lastly, we evaluated if the timing of neonatal priming would result in different outcomes in astrocytic ($GFAP^+$) (**Fig. 4.8B**) and microglial ($Iba1^+$) (**Fig. 4.8C**) proliferation. Animals that were primed during the first neonatal week depicted a similar astrocytic proliferation of an adult acute response, but a lower microglial response in the pons and the cerebellum was observed. Interestingly, these are the main regions affected both by lasting and primed neuronal demise.

Regarding the animals primed during the second week, lower numbers of astrocytes and microglia were present in the hippocampus. The cerebellum of these animals depicted a slightly higher number of microglial cells than in an acute response. The differences to priming in the first week may hint on the interactions involved in the neuronal demise observed in this study.

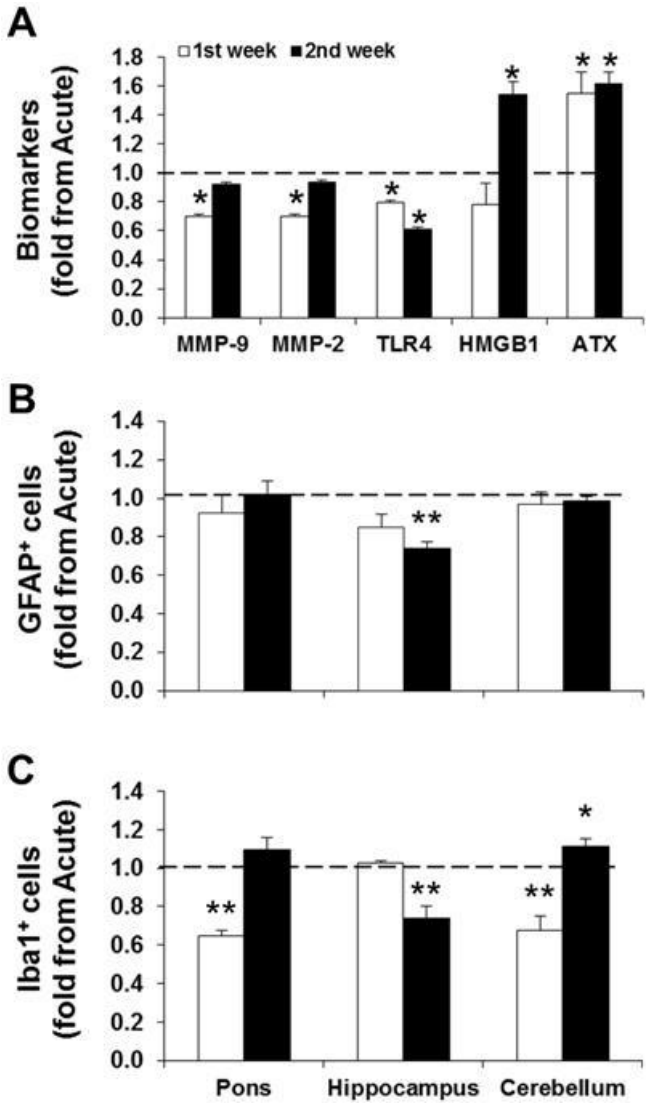


**Figure 4.8 - Inflammatory response of animals primed in the first week of life is milder than in animals primed during the second week.** In A, whole brain lysates of young adult mice, with lipopolysaccharide (LPS)-administration in the first or second weeks of life followed by a second LPS-challenge at adult age, were used to determine the activity of matrix metalloproteinase (MMP)-9 and MMP-2 by gelatin zymography. The expression of inflammatory biomarkers Toll-like receptor (TLR)-4, high-mobility group box (HMGB)-1 and autotaxin (ATX) were quantified by western blot. Paraffin sections from the same mice were immunolabelled for astrocytes (glial fibrillary acidic protein, GFAP, red) and for microglia-like cells (ionized calcium binding adaptor molecule, Iba1, green). Nuclei were counterstained with DAPI (blue). Number of $GFAP^+$ cells (B) and of $Iba1^+$ cells (C) in the parenchyma were determined per field. All results are fold-change from respective adult acute response ± SEM of at least four independent experiments. *P <0.05 and **P <0.01 *vs*. adult acute response.

## 4. Discussion

In the present study, we establish the acute, lasting and primed responses of neonatal systemic inflammation. We demonstrate that the neonatal acute response of neurons and inflammatory mediators varies with the age at which LPS exposure occurs. We also demonstrate that an inflammatory episode during the first week of life has more lasting effects on neurons, whereas it alters lastly both white matter and astrocytes if the induction occurs during the second week. At last, we provide novel insights into the primed responses at both neonatal ages, again revealing a more neuron-related demise in animals primed in the first week, and a more glial response for the second week priming.

We observed that LPS administration during the second week of life did not alter body and brain weights. Another study that studied the effect of LPS administration (0.3 mg/kg) on newborn mice from PND3 to PND11 (Du et al. 2011), revealed that body weight is maintained at PND12 and thus consistent with our results. Regarding the body weight loss seen in primed young adults following a second LPS-challenge (though it was only significant compared to the adult acute response in animals with priming during the first week of life), both groups had significant weight loss when compared to controls with only SF administration. In line with our results, another study has reported that a second injection of LPS induces weight loss in primed animals regardless of the neonatal week of the first injection (Spencer et al. 2007). As for the fact that one single dose of LPS in adulthood induced weight loss in animals that received SF during the second week of life but not during the first, Avitsur and Sheridan (2009) have studied the influence of maternal separation at PND4 or PND9 on the adulthood response to LPS-administration. This work concluded that the mere handling of the pups may actually be an inductor of stress, particularly in the 2 week of life and thus similarly to our study. To point that, even though our SF-treatment pups were separated from dams for short periods of time, it was through three consecutive days for it may further contribute to this outcome.

We further determined the effects on neurons and white matter. Our results have shown neuronal loss and atrophy at PND14, as well as reduction of the white matter in cerebellum. In consistence with our data, the aforementioned study of LPS administration from PND3 to PND11 also demonstrated neuron loss at PND12, in addition to myelin loss (Du et al. 2011). Oligodendrocyte precursors (OPC) are vulnerable to LPS exposure during the perinatal period, leading to the arrest of their maturation (van den Heuij et al. 2014). A recent study (Bénardais et al. 2014) has also revealed that animals previously injected with LPS (0.2 mg/kg) in the first neonatal week have delayed myelin removal in the corpus callosum when it was induced at the

age of 8 to 9 weeks, along with enhanced early remyelination. Moreover, exposure of preterm sheep to LPS reduced neuroinflammation and white matter injury after a final dose 10 days later (van den Heuij et al. 2014). Still, *in vitro* studies have showed that inflammation may also injure OPC leading to maturation-dependent cell death and loss of cellular processes (Back et al. 1998; Oka et al. 1993). Thus, the observed reduction of white matter may be related to the particular vulnerability of oligodendrocytes and OPCs. Again, the difference between the groups that received one dose of LPS at adulthood and one dose SF either at the first or at the second weeks may be due to dam's separation.

Our current data further revealed that LPS-administration during the first week of life does not induce enduring alterations in the cerebellar layers of adults. Indeed, the opposite was only observed in young adults subjected to LPS-treatment during the second week of life, namely in the white matter layer. As for the neonatal priming, animals that were primed during the first week of life had decreased molecular, Purkinje and internal granular layers. Those primed in the second week had further reduction of the cerebellar white matter layer and of the internal granular layer. The decrease in both molecular and purkinje layers could be related to the loss of Purkinje neurons. In agreement with our data, white matter injury has been formerly connected to neurodevelopmental damage in neonatal sepsis survivors, involving the production of pro-inflammatory cytokines, BBB disruption and impaired autoregulation of cerebral blood flow (Graham et al. 2004; Khwaja and Volpe 2008; Volpe 2008). The narrowing of the internal granular layer could be related to impairment of granule cells proliferation. This process occurs mostly during the first two weeks of life, more precisely between PND5 and PND10 (Xu et al. 2013). As so, these cells might be particularly vulnerable to subsequent LPS-induced damage.

We further evaluated the acute and enduring effects of neonatal inflammation on neurons neuronal development. We determined that exposure to LPS during the first week of life induced lasting neuronal atrophy in young adults, with no further damage after a second exposure (primed animals). Other studies where systemic inflammation was induced in the first week after birth also had no overall adult neuronal loss (Cai et al. 2013; Fan et al. 2011). Still, the animals presented motor function deficits and loss of dendrites. Similarly, a study in adult mice reported that LPS affected neurogenesis giving rise to neurons with less synaptic contacts along with decreased dendritic volume and complexity (Valero et al. 2014). Moreover, neonatal inflammation has been revealed to specifically affect dividing neurons but not post-mitotic cells (Järlestedt et al. 2013). It is conceivable that the hippocampal and brainstem lesions here demonstrated may underline the long-term cognitive disorders observed in SAE

survivors (Sonneville et al. 2013). As for late neonatal exposure to LPS, our data revealed an acute neuronal loss in the pons by double LPS challenge (primed acute at PND53), as well as neuronal atrophy in the pons (enduring at PND53) and hippocampus (primed acute at PND53). The work by Järlestedt et al. (2013) demonstrated reduced number of neurons at PND41, 32 days after LPS, though in the hippocampus. Still, we only observed neuronal atrophy in this region. This divergence could be due to the exact days of neonatal administration, as our pups were exposed to LPS at days 11, 12 and 13 – later than PND9 as in his study –, and we only observed enduring neuronal loss in the hippocampus of pups injected at days 4, 5 and 6. Overall, lasting effects on neurons are more severe in young adult mice with inflammation during the first week of life, whereas the primed acute effects are more harmful in animals with inflammation during the second week of life. Thus, permanency of type of cells and regions impaired seem to depend on the neonatal age at which systemic inflammation is induced (Pandharipande et al. 2013).

Neuronal apoptosis can originate from the subsequent production of inflammatory mediators by sepsis-induced activation of astrocytes and microglia (Liu et al. 2001). The acute response of MMPs to LPS administration during the second week of life are consistent with our own previous results still unpublished on neonatal inflammation during the first week of life (Chapter 3), were we demonstrated an increased the activity of MMP-9 with no changes in MMP-2. Tough HMGB1 was overexpressed, the same was not observed for TLR4. This may be intricately related to the fact that ATX expression was also not altered. Regarding the response of adult TLR4 to a second exposure, it was lower than in adult acute inflammation regardless of the neonatal period at which the first exposure was inflicted. A study by Murray et al. (2012) demonstrated that animals with inflammation during the first week of life displayed no alterations in TLR4 expression when treated with LPS as adults. These mechanisms for LPS tolerance maintains TLR4 expression suppressed for over 24 h (Bosisio et al. 2002; Nomura et al. 2000). TLR4 is intimately connected to cyclooxygenase-2 (COX-2), given that the activation of the first leads to the production of the last (Parente et al. 2013). Given this relationship, an attenuated fever response by decreased COX-2 expression may result from similar TLR4 decreased expression and vice-versa (Karikó et al. 2004; Kis et al. 2006; Spencer et al. 2011). A few studies state that neonatal LPS exposure at PND14 attenuates LPS-induced fever in adults (Ellis et al. 2005; Spencer et al. 2006) and thus also in line with our data on young adults primed during the second week. The response of HMGB1 and ATX to a second systemic LPS administration in adults are usually elevated for a few days (Awada et al. 2012; Chavan et al. 2012; Terrando et al. 2010), as seen in mice that were primed during the second

week of life. There are no further studies on the influence of neonatal inflammation in the adult expression of HMGB1 that could justify the difference from its priming at the first or second weeks of life. Still, it is important to bear in mind that this molecule has a late kinetic activity (Goto et al. 2010; Kato et al. 2009; Nagamori et al. 2012) and, thus, it may still be overexpressed at other time-points in young adult animals primed during the first week of life.

Inflammatory mediators could be produced by activated microglia and astrocytes, an event that has been described in rat models of SAE and in humans (Deng et al. 2013; Lemstra et al. 2007). Microglial activation is one of the earliest changes observed in SAE (Nayak et al. 2014; Semmler et al. 2005). Interestingly, the microglial numbers in the adult animals here studied were alike in general. A recent study has demonstrated that exposure to LPS at PND7 or at PND21 trigger similar microglial densities, suggesting that there may be a maximum number of microglia allowed to proliferate in the brain (Smith et al. 2014). Still, it has been demonstrated that LPS-administration during the first week of life produces more activated microglia in response to a second challenge (Bland et al. 2010). We, however, observed the opposite with more microglia than in an adult acute response in the cerebellum of animals primed during the second week of life.

Microglial activation may affect other brain cells such as astrocytes and neurons (van Gool et al. 2010). Though our study depicted similar numbers of an adult acute response, the increase in $GFAP^+$ astrocytes is associated with SAE in rat models in order to repair the injured CNS (Rezaie and Dean 2002; Semmler et al. 2008). Also to point that preterm sheep with administrated LPS present astrocytosis following a second dose (van den Heuij et al. 2014). In our study, the response of adult astrocytes to a second stimulus appears to be deeply connected to the neonatal week of the first exposure. Surprisingly, mice primed during the second week had decreased numbers of $GFAP^+$ cells in response to a second pro-inflammatory induction. Recent studies have demonstrated that early inflammation triggers acute and brain alterations, with significant astrogliosis and microglial activation in LPS-exposed adult mice (Cai et al. 2013; Dada et al. 2014; Fan et al. 2011; Murray et al. 2012). Still, Järlestedt et al. (2013) have recently demonstrated that LPS administration at PND9 leads to reduced numbers of astrocytes in young adult animals, even without a second exposure, as seen in our data. In addition, even though there is generally less microgliosis/proliferation in the primed mice following the double LPS challenge, microglia may still be activated (Bland et al. 2010). This has been revealed to diminish the density of astrocytes and increase neuronal damage (Sonneville et al. 2012), coinciding with our results. The morphology of hippocampal astrocytes in the neonatal

period may also influence the final outcome in young adults. At PND7 their processes overlap, whereas around PND14 these are trimmed giving cells a mature appearance (Bushong et al. 2003; Bushong et al. 2004; Freeman 2010). Therefore, the inability to repair inflammatory damage in the first week of life might prime these cells for future responses, whereas the same might not happen in the second week of the developing CNS.

## 5. Conclusions

In conclusion, the present data revealed that neonatal systemic inflammation induces not only enduring effects in young adults but also influences the response to future inflammatory exposure. More importantly, we hereby demonstrate that the neonatal window of neuroinflammation determines the type of cell and region responses in the CNS and thus its neurodevelopmental outcome. Further studies need to be done in order to further identify the permanent damaging effects in the CNS, as well as the mechanisms behind the differential response associated with the developmental window of CNS development after birth, opening new target-driven medicines and hopefully suppressing long-term sequelae in patients after neonatal sepsis.

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*Chapter 5*

# CONCLUDING REMARKS

## 1. Concluding Remarks

The deleterious effects of UCB and lipopolysaccharide LPS to brain cells, either alone or in combination, have already been examined in our previous studies (Falcão et al. 2005; Fernandes et al. 2004; Silva et al. 2010; Veszelka et al. 2007). In addition, it has been demonstrated that astrocytes are particularly sensitive to these molecules (Falcão et al. 2005; Fernandes et al. 2004). Despite this, little was known about the aggravating effects of UCB and/or LPS on the BBB dynamic properties. This led us to evaluate the effects of UCB and LPS on a simplified *in vitro* model of the BBB formed by rat BMEC monocultures, as well as to assess the consequences of astrocytic reactivity on BBB integrity using co-cultures of rat BMEC and astrocytes. After understanding the in vitro effects induced by these two insults that are common in neonatal conditions, we performed *in vivo* studies to better evaluate glial response and its influence on neurodevelopment, using LPS as a trigger of neuroinflammation in a newborn mouse model. We investigated the influence of LPS administration during the first week of life in the developing NVU. Thereafter, we evaluated the acute effects of LPS during the second week of life, the lasting effects in young adult animals resulting from exposure to the inflammatory stimuli in the neonatal period, as well as the primed response in a second stimulus at young adulthood (Fig. 5.1).

In the first study (**Chapter 2**) we demonstrated that both LPS and UCB induce apoptosis in BMEC after 24h, though only UCB triggered LDH release. The inhibition of P-gp activity by these molecules occurs at different time-points, as LPS effect was earlier and transient whereas it is prolonged for UCB. Considering that UCB is a substrate of P-gp (Sequeira et al. 2007), our findings hint that P-gp might not have the capacity to export both UCB and R123 simultaneously. By further assessing the integrity of the BMEC monolayer, we observed that MMPs activity, BMEC permeability and TEER were especially altered at earlier time-points, reaching a peak of impairment. Overall, damage to organelles by UCB was shown to be more remarkable than that triggered by LPS, as confirmed by ultrastructural analysis. This can be related to alterations in the dynamic properties of cellular membranes upon interaction with UCB (Brito et al. 2001; Rodrigues et al. 2002). The effects of LPS in mono-cultures permeability and TEER become less marked with the incubation duration, since this molecule would only directly interact with TLR4 of BMEC in the first hours of exposure (Singh and Jiang 2004). UCB, on the other hand, has resulted in an increased toxicity. This appears to be connected with the fact that TJs are hardly visible in UCB-treated BMEC, probably facilitaing the paracellular passage of UCB across the endothelium. Thus, UCB might also the release of soluble factors when BMEC are co-cultured with astrocytes, that may further alter the expression and localization of TJ as well as of AJ.

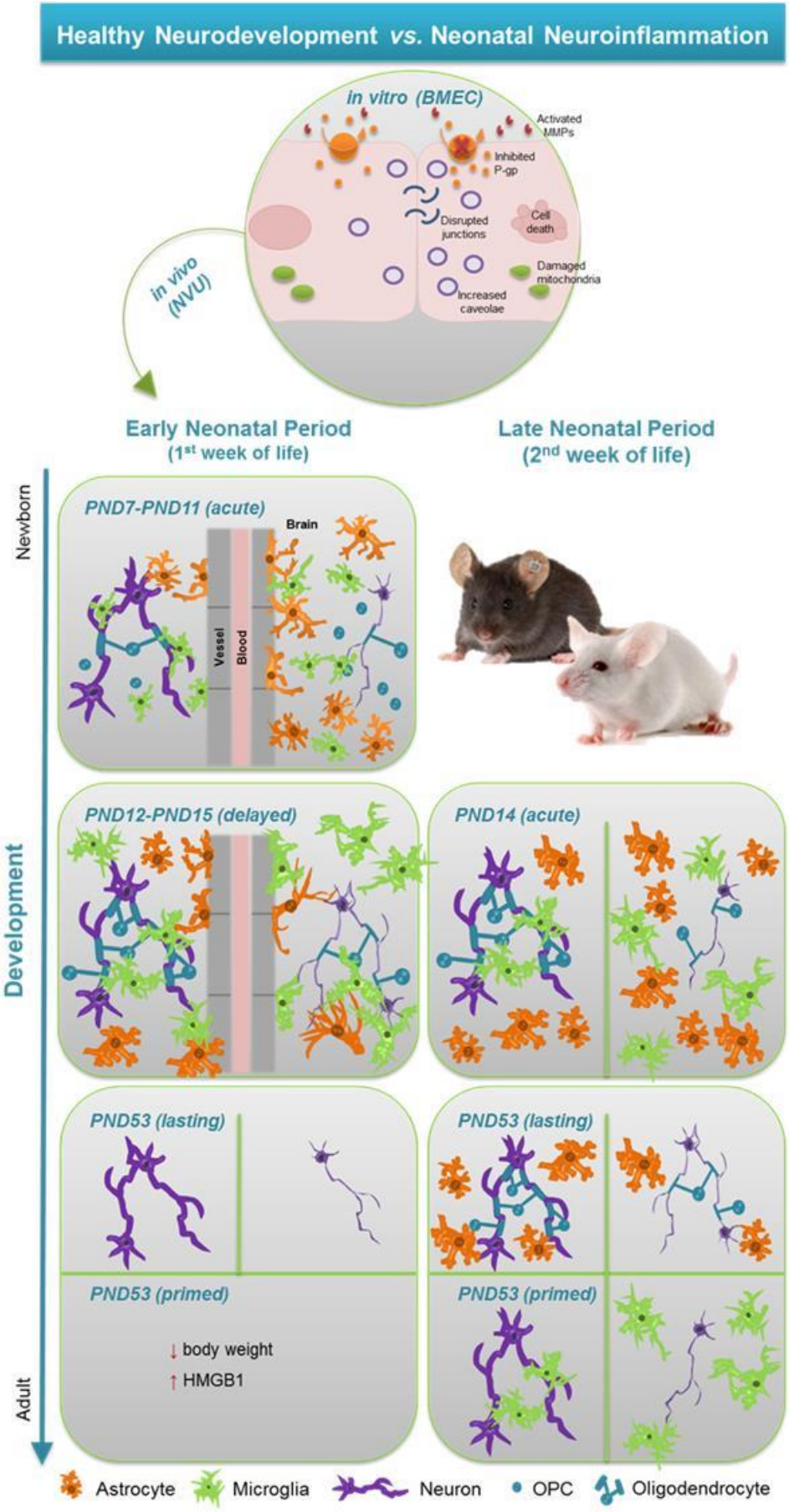
Healthy Neurodevelopment vs. Neonatal Neuroinflammation
in vitro (BMEC)
Activated MMPs
Inhibited P-gp
Disrupted junctions
Cell death
Damaged mitochondria
Increased caveolae
in vivo (NVU)
Early Neonatal Period
(1st week of life)
Late Neonatal Period
(2nd week of life)
Newborn
Development
Adult
PND7-PND11 (acute)
Brain
Vessel
Blood
PND12-PND15 (delayed)
PND14 (acute)
PND53 (lasting)
PND53 (lasting)
PND53 (primed)
↓ body weight
↑ HMGB1
PND53 (primed)
Astrocyte
Microglia
Neuron
OPC
Oligodendrocyte

**Figure 5. 1 - Schematic representation of the major findings achieved in the present work.** Studies performed in vitro in primary cultures of rat brain microvascular endothelial cells (BMEC) showed that common neonatal inflammatory stimuli, like unconjugated bilirubin (UCB) and lipopolysaccharide (LPS), lead to cell death and damaged organelles. In addition, these molecules increase the number of caveolae, inhibit the activity of P-glycoprotein (P-gp), and compromise the integrity of the intercellular junctions. Our in vivo studies on neonatal sepsis further showed that the timing of chronic systemic inflammation determines not only the lasting effects but also the primed response of the neurovascular unit (NVU) to other inflammatory episodes. Indeed, the induction of inflammation in the first neonatal week leads to neurodegeneration and with no further neuronal damages after a second episode. On the other hand, if the neonatal inflammatory response occurs in the second week of life, the young adult animals present not only neurodegeneration, but also lower levels of myelin and fewer astrocytes. Surprisingly, a primed response triggers neuronal loss and microglia's proliferation/migration though only in animals that sustained neuroinflammation during the second week of life. OPC, oligodendrocyte precursor cell; PND, postnatal day.

In addition, despite astrocytes being able to strengthen BMEC barrier properties (Cardoso et al. 2010; Deli et al. 2005; Descamps et al. 2003; Toth et al. 2011; Veszelka et al. 2007), their inflammatory response (Didier et al. 2003) may actually worsen the effects of LPS through accumulation not only of β-catenin in the perinuclear region, but also of ZO-1 that had never been reported previously. Altogether, this chapter revealed that each compound has its own mechanism of action and temporal damaging profile. Events occur earlier and transiently in LPS-treated cells, thus not aggravating in its majority the UCB-induced cell demise. Surprisingly, the presence of glial cells alters these profiles and further contributes to BBB disruption.

Afterwards (**Chapter 3**), we demonstrated that neonatal repeated administration of LPS injections lead to prolonged cerebellar hypoplasia which seems to be directly associated with the thinning of cerebellar layers. This may also be connected to the neuronal loss and decreased soma area observed in all studied regions. The surprising decrease in autotaxin's expression confirmed its important role in oligodendrocyte process network formation and expansion (Dennis et al. 2008; Yuelling et al. 2012). Indeed, myelination was already delayed at this time-point, with a parallel increase of OPC whose maturation was possibly arrested. The present results revealed different MMP-2 activity from our studies in **Chapter 2**, probably due to the fact that this enzyme can be deactivated *in vivo* but not *in vitro* (Mizoguchi et al. 2008). Also, LPS triggers a late pro-inflammatory response (Goto et al. 2010; Mun-Bryce et al. 2002; Nagamori et al. 2012) that is sustained up to one day, namely by microglia (Shimazu et al. 1999; Takata et al. 2011). The co-localization of microglia and astrocytes with the microvasculature in response to LPS may be a protective attempt to reduce BBB permeability (Kaya et al. 2004). Astonishingly, the hypertrophic and intermediate-

activated morphologies of microglia coincided with the decrease in $GFAP^+$ cells. Microglial activation modulates astrocytic proliferation and vice-versa (Seifert et al. 2006). Thus, microglial transformation into these morphologies could either cause the loss of $GFAP^+$ cells, or be in fact induced by signals released from astrocytes, like CX3CL1 (Hulshof et al. 2003). Collectively, these data show that glial cells may be intimately involved in the LPS-induced neuronal and myelination atrophies, given that the physical association between these cells is essencial for proper synapse and myelin formation (Ji et al. 2013; Miyamoto et al. 2013; Moore et al. 2011; Pang et al. 2013). Moreover, it also indicates that $GFAP^+$ cells could in fact be a result of astrocytic reprogramming into NPC, repopulating the neuronal lineage (Corti et al. 2012; Heinrich et al. 2010; Yang et al. 2012) or even giving rise to microglia (Elmore et al. 2014).

In our final experimental approach we determined the acute effects of systemic inflammation during the second week of life. In addition, we evaluated the lasting effects of neonatal systemic inflammation on neurodevelopment in young adult animals, as well as whether differences were determined by the neonatal period of neuroinflammation onset. Moreover, we determined if the priming of the inflammatory response during the neonatal period caused alterations in the adult inflammatory response to a second LPS-stimulus, compared to a single injection at adulthood (adult acute response) (**Chapter 4**). These studies demonstrated that neuroinflammation during the second week of life did not induce loss of body and brain weights, which is consistent with other studies on neonatal inflammation (Du et al. 2011). Still, these animals presented acute neuronal loss and atrophy (as seen in **Chapter 3**), as well as shrinkage of the cerebellar white matter. Other studies have shown that myelin is particularly vulnerable during the perinatal period (van den Heuij et al. 2014) which, along with the neuronal atrophy here observed, could justify this result. Moreover, there was increased acute astrogliosis and microgliosis, in agreement with other models of SAE (Deng et al. 2013; Lemstra et al. 2007). Our studies (**Chapter 4**) further demonstrated that the administration of LPS during the first week of life did not induce lasting effects on the cerebellar layers of young adult animals, nor did it exacerbate/protect the response of an adult acute response to LPS insult. On the other hand, in animals with LPS administration during the second week there was sustained white matter reduction, which was exacerbated by a second stimulus at young adult age in addition to a decrease in the internal granular layer. Therefore, the white matter damage is more severe in animals with systemic inflammation during the second week of life, probably due to a greater vulnerability of oligodendrocytes and their precursors (Back et al. 1998; Oka et al. 1993). The restoration of myelin at adulthood in animals treated with LPS during the first week of life may be due to the process of OPC

maturation being only delayed (**Chapter 3**), whereas its loss in those treated during the second week could possibly be related to their death. As for neuronal demise, this appears to be significant in animals with systemic inflammation during the first week of life, given that these animals have lasting neuronal atrophy and loss in all brain regions examined. This is consistent with previous studies suggesting that the neonatal age at which inflammation is induced determines the cell type and the region that will be affected (Pandharipande et al. 2013). Our data also revealed that the time-point of neonatal priming determines the adult response of HMGB1, whereas the expression of TLR4 is always lower in animals with priming in either neonatal week compared to an adult acute response. Inflammation during the first week followed by a second exposure in young adults does not exacerbate HMGB1 expression, though it is significantly increased in young adults when priming occurs during the second week. As for the unresponsiveness of TLR4, this could be due to a primed tolerance that suppresses its activation for 24h (Bosisio et al. 2002; Nomura et al. 2000). Given that microglial activation and neuronal damage are associated with altered TLR4 expression (Murray et al. 2012) we would expect an attenuated glial response. Indeed, we observed a decrease in $Iba1^+$ cells proliferation/migration in animals primed during the first week, but not in those primed during the second week. This suggests that astrocytes and microglia may have been primed by an ENP inflammation and, given the overall impact of this second response, it may exert a protective effect. Overall, this chapter revealed that neonatal systemic inflammation induces long-term neuronal and myelin damage and influences future inflammatory responses, with distinctive outcome depending on the neonatal age of the inflammatory onset.

In conclusion, this thesis enlightened many fundamental questions on the interactions within the NVU in response to common problematic clinical conditions in the neonatal period. We were able to demonstrate that these conditions alter BBB permeability by the disruption of intercellular junctions and degradation of ECM. In addition, the direct and indirect stimulation of astrocytes further disrupts BBB integrity, and may as well modulate the progression of microglial maturation. Ultimately, the inflammatory response within the developing NVU leads to lasting neuronal and white matter atrophies, which severity depends on the neonatal age of the inflammatory injury. Still, other important questions were raised in this thesis, namely the true role of astrocytes and microglia in the neuronal outcome after inflammation: demising or repairing? Therefore, more studies are needed in order to better understand the differences between early and late neonatal neuro-glia-vascular inflammatory responses, so as to modulate them accordingly by using more directed and specific therapeutic approaches.

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